Human Eccrine Sweat Gland Cells Can Reconstitute a Stratified Epidermis

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Für meine Eltern

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Abbreviations

BPAG	bullous pemphigoid antigen
BSA	bovine serum albumin
CD	cluster of differentiation
DAPI	4',6-diamidino-2-phenylindole
DIG	digoxigenin
DNA	deoxyribonucleic acid
Dsg	desmoglein
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
FGF	fibroblast growth factor
FITC	fluorescein isothiocyanate
HMB-45	human melanoma black
IF	intermediate filament
K	(cyto-)keratin
KdES	keratinocyte derived epidermal substitutes
MMP	matrix metalloproteinase
mTOR	mammalian target of rapamycin
Na-ATPase	sodium-potassium pump
PAR-2	protease activated receptor 2
PAS	periodic acid-Schiff
PCR	polymerase chain reaction
PDGF	platelet derived growth factor
PFA	paraformaldehyde
RGM	Rheinwald and Green medium
SdES	sweat gland derived epidermal substitutes
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SPR	small proline rich
TG	transglutaminase
TGF-β	transforming growth factor β
TRADD	tumor necrosis factor receptor 1 associated death domain protein
TRITC	tetramethyl rhodamine isothiocyanate
UV	ultraviolet

Summary

The major goal of this work was to investigate whether human eccrine sweat gland cells, in analogy to cells of the hair-follicle bulge, exhibit the capacity to develop into a functional epidermis. Conclusive results towards this question were not reported so far.

To address this issue, I generated dermo-epidermal skin substitutes in vitro. All skin substitutes were based on collagen hydrogels, containing human primary dermal fibroblasts within the gel, and eccrine sweat gland cells on the gel (or epidermal keratinocytes as controls). Skin substitutes generated in vitro were transplanted onto the backs of immuno-incompetent rats and the expression of epidermal markers, indicative of the grade of homeostasis and differentiation, was determined. In addition, potential stem cell markers were tested. I was able to demonstrate both, in vitro and in vivo, the capability of human eccrine sweat gland cells to form a stratified interfollicular epidermal substitute on collagen hydrogels (Biedermann, 2010). This was substantiated by the following findings: (1) A stratified epidermis consisting of 10-12 cell layers is formed by sweat gland cells. (2) A distinct stratum corneum develops and is maintained after transplantation onto immuno-incompetent rats. (3) Indicative proteins, such as filaggrin, loricrin, involucrin, envoplakin, periplakin, and transglutaminases I and III, are matching the characteristic expression pattern of normal human skin. (4) Junctional complexes and hemidesmosomes are readily and regularly established. (5) Cell proliferation in the basal layer reaches homeostatic levels. (6) The sweat gland derived epidermis is anchored by hemidesmosomes within a well developed basal lamina. (7) Palmoplantar or mucosal markers are not expressed in the sweat gland derived epidermis. These data suggest that human eccrine sweat glands are an additional source of keratinocytes that can generate a stratified epidermis. Based on these findings a novel question arises, namely as to which extent human skin is repaired and/or permanently renewed by eccrine sweat gland cells.

I am presently determining if sweat gland cells forming an epidermis are capable to take up melanin, produced by melanocytes (Biedermann, in preparation). If so, this would prove that sweat gland cells are indeed fully differentiated epidermal keratinocytes, because this characteristic was never observed before to be a property of eccrine sweat gland cells.

Zusammenfassung

Das Hauptziel dieser Arbeit war es, nachzuweisen, ob humane ekkrine Schweissdrüsenzellen, in Analogie zum Haarfollikel-Bulge, die Fähigkeit besitzen, eine Epidermis zu bilden. Eindeutige Resultate hinsichtlich dieser Fragestellung wurden bisher nicht gezeigt.

Ich habe dazu dermo-epidermale Hautsubstitute hergestellt. Diese Hautsubstitute basieren auf Kollagenhydrogelen, die humane primäre dermale Fibroblasten im Gel enthalten und auf deren Oberfläche ekkrine Schweissdrüsenzellen (oder aber normale epidermal Keratinozyten – als Kontrollzellen) aufgebracht wurden.

Die auf Vollhautwunden immun-inkompetenter Nacktratten transplantierten Substitute wurden auf epidermale Marker der Gewebshomeostase und der Hautdifferenzierung, aber auch bezüglich potentieller Stammzellmarker getestet.

Ich konnte nachweisen, dass humane ekkrine Schweissdrüsenzellen sowohl in vitro als auch in vivo fähig sind, eine stratifizierte interfollikuläre Epidermis auf Kollagen-Hydrogelen zu bilden (Biedermann, 2010). Dies wird durch folgenden Befunde untermauert: (1) Eine stratifizierte Epidermis, bestehend aus 10-12 Zellschichten, wird durch Schweissdrüsenzellen gebildet. (2) Es entwickelt sich ein ausgeprägtes Stratum Corneum, das auch nach Transplantation auf immuninkompetente Ratten vorhanden ist. (3) Proteine wie Filaggrin, Loricrin, Involucrin, Envoplakin, Periplakin, Transglutaminase I und III zeigen das gleiche charakteristische Expressionsmuster wie in normaler humaner Haut. (4) Junctionale Komplexe und Hemidesmosomen werden etabliert. (5) Die Zellproliferation in der basalen Schicht erreicht ein homoestatisches Niveau. (6) Die aus Schweissdrüsenzellen resultierende Epidermis ist durch Hemidesmosomen auf einer gut entwickelten Basallamina verankert. (7) In der aus Schweissdrüsenzellen gebildeten Epidermis sind keine palmo-plantaren oder mukosalen Marker vorhanden. Diese Resultate sind ein starker Beleg dafür, dass Schweissdrüsen eine zusätzliche Quelle für epidermale Keratinocyten sind, die eine stratifizierte Epidermis bilden können. Die gewonnenen Erkenntnisse erlauben nunmehr die weiterführende Frage, in welchem Ausmaß ekkrine Schweissdrüsenzellen fähig sind, die humane Haut zu reparieren und/oder sie ständig zu erneuern.

Gegenwärtig versuche ich nachzuweisen, ob Schweissdrüsenzellen, die eine Epidermis bilden, Melanin aufnehmen können, welches Melanocyten produziert haben (Biedermann, in preparation). Dies würde zusätzlich unterstreichen, dass

Schweissdrüsenzellen voll funktionsfähige epidermale Keratinocyten sind, da dies eine Eigenschaft ist, die nicht bei Schweissdrüsenzellen, die als solche in der Schweissdrüse funktionieren, beobachtet werden kann.

1 Introduction

1.1 Structure and function of human skin

As the largest organ of the body and the interface between the body and the environment, the skin plays a fundamental role in protecting and maintaining the internal milieu of humans. Moreover, the skin is responsible for mediating immune reactions and sensory input. The skin consists of the superficial ectodermally derived epidermis, the dermis originated from the mesoderm and the subcutaneous fat tissue, the hypodermis (Fig. 1). The total surface of the skin of a human adult is about 1.5 - 2 m^2 and the skin weights about 1/6 of the total body weight. Depending on the site of the body its thickness varies between 1.5 – 4 mm, not including the hypodermis. Keratinocytes are the main cell type in the epidermis; other less frequent cells are melanocytes, Langerhans and Merkel cells. The basement membrane separates the epidermis from the underlying dermis. Fibroblasts are the dominating cell type in the dermis, producing extracellular matrix components such as elastic fibres, collagenous fibres and reticular fibres. Furthermore, the dermis contains blood vessels in the papillary and reticular compartments for nourishment. Importantly, the epidermis is supported via diffusion from the dermal connective tissue.
In addition, skin appendages and adnexal structures, like hair follicles, sebaceous glands, sweat glands and nails are situated in the human skin.

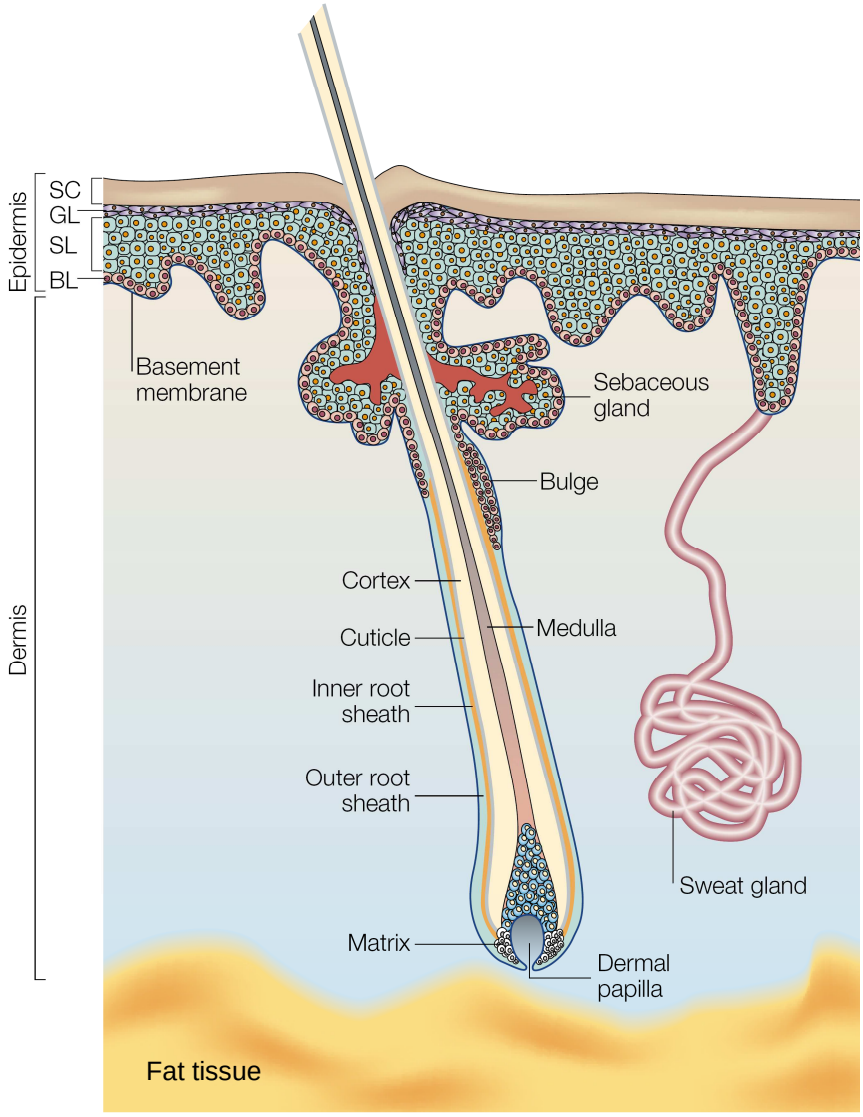

Figure 1: Epidermis and skin appendages. The epidermis, the dermis and hypodermis (fat tissue) compose the skin. Skin appendages such as hair follicles, sebaceaous and sweat glands reside in the dermis. (BL: basal layer, SL: spinous layer, GL: granular layer, SC: stratum corneum) (Fuchs, 2002).

1.2 The Epidermis

1.2.1 Structure and function of human epidermis

The outermost surface of the body is the stratified squamous epidermis. This part of the skin is the first barrier against environmental influences. It protects against physical and chemical environmental influences, such as UV-light or against microbial assaults like bacteria or fungi (Proksch, 2008). Moreover the epidermis is important to prevent dehydration, protein and electrolyte loss. The epidermis consists mainly of keratinocytes (Fig. 2) and among them reside pigment producing melanocytes, Langerhans cells as cells of the immune system (Merad, 2008) and touch sensing Merkel cells (Halata, 2003, Moll, 2005, Lucarz, 2007). The innermost layer of the epidermis, the basal layer (stratum basale), rests on the basement membrane (Freinkel, 2001). Keratinocytes in this layer proliferate, giving rise to more differentiated cells of the stratum spinosum, granulosum and corneum. The outermost layer, the stratum corneum, consists of terminally differentiated (dead) cells.

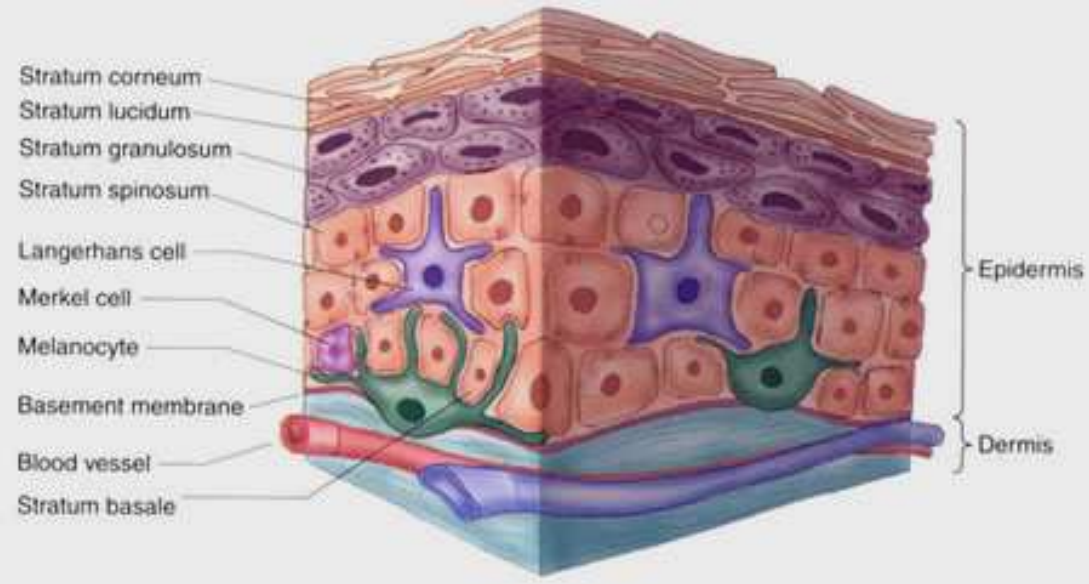

Figure 2: Structure of human interfollicullar epidermis. The epidermis consists of keratinocytes, melanocytes, Langerhans and Merkel cells. (http://www.med-ed.virginia.edu/courses/cell/handouts/images/Skin2.jpg).

1.2.2 Keratinocytes

As keratinocytes (Fig. 3) are the main cell type of the epidermis they are responsible of replenishing the epidermis throughout life. There is a dynamic state between keratinocyte stem cells, progeny cells, more differentiated cells and terminally differentiated cells (Blanpain, 2009). One epidermal keratinocyte stem cell has the capacity to give rise to enough cells to cover the body surface (Rochat, 1994, Watt, 2000).

The progeny cells of the stem cells, residing in the basal layer, are referred to as transit amplifying cells (Jones, 1993, Jones, 1995). Transit amplifying cells are rapidly dividing cells that proliferate until they withdraw from the cell cycle starting their terminal differentiation. For differentiation, keratinocytes detach from the basement membrane by losing their anchorage by hemidesmosomes (Barrandon, 1987, Watt, 1998). They enter the spinous layer and modify their cytoskelatal composition and intercellular connections. Important intercellular adhering junctions are desmosomes. They serve to attach neighbouring cells to each other. Desmosomes are found predominantly in the epidermis and the heart (Green, 2000). Functionally, they consist of three families of proteins. To these belong cadherins (desmocollins and desmogleins), plakins (desmoplakin) and armadillo proteins (plakoglobin and plakophilins). The localization of desmogleins and plakophilins varies in the epidermis, whereas proteins like plakoglobin and desmoplakin are expressed throughout the epidermis. As example, desmoglein 1 (Dsg1) is found in the upper epidermal layers. High expression levels of desmoglein 3 (Dsg3) are detected in lower layers (Kottke, 2006). The differential expression pattern in the epidermis could play a role in tissue morphogenesis. Desmoglein 1 normally promotes terminal differentiation of keratinocytes in upper epidermal layers, whereas Desmoglein 3 might function in proliferation of keratinocytes. This was confirmed by the observation that overexpression of desmoglein 3 caused hyperproliferation in upper epidermal layers of transgenic mice (Merritt, 2002).

Desmosomes are anchored to the intermediate filament cytoskeleton (Green, 2007). Hence, desmosomes provide rigidity and strength to keratinocytes. This function was confirmed by studies showing that an autoantibody against desmoglein 3 destroyed the desmosomes causing the skin disease pemphigous vulgaris (Jones, 1986, Amagai, 1991, Payne, 2004).

As keratinocytes enter the granular layer they produce proteins necessary to establish the epidermal barrier. Tight junctions are most important for the constitution of the epidermal water barrier. They also regulate the paracellular flux of water-soluble molecules between adjacent cells (Turksen, 2002). The main structural proteins of tight junctions are claudins and occuldins. There exist approximately 24 subtypes of claudins. Each of the claudins displays a unique tissue expression pattern. In the epidermis the main claudins are claudin 1 and 4 (Furuse, 2006). Claudins also bind to other membrane macromolecules, including the zona occludens (ZO) proteins ZO-1, ZO-2, ZO-3, and multi-PDZ domain protein-1 (Anderson, 2004, Niessen, 2007). The ZOs provide a direct link to the actin cytoskeleton of keratinocytes.

In the stratum corneum the cells have turned into dead flattened squames constituting the outer protective cell layer.

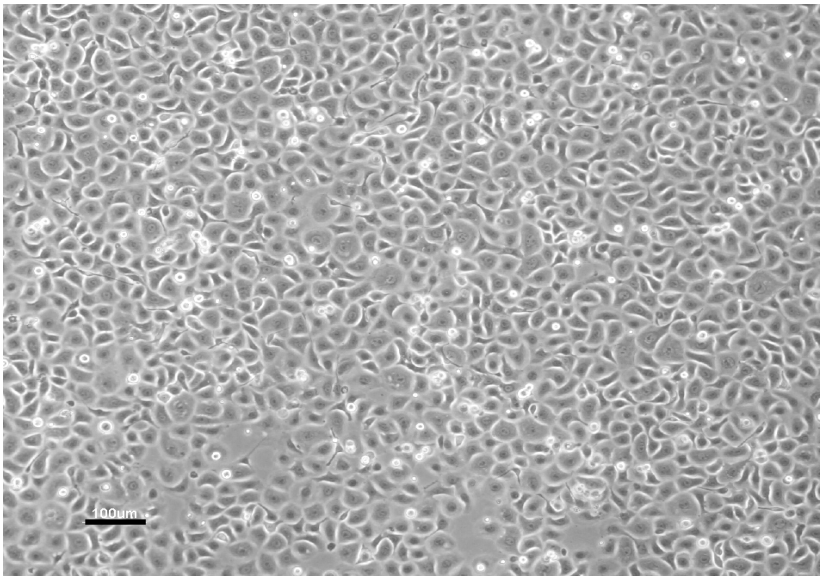

Figure 3: Human primary keratinocytes in culture. These keratinocytes were isolated from a human skin biopsy and cultured on cell culture plastic. (bar, 100 µm).

1.2.2.1 Cytokeratins

Eucaryotic cells contain three major types of cytoskeletal proteins: microtubles, actin filaments (microfilaments) and intermediate filaments (Fig. 4).

Keratins belonging to the group of intermediate filaments are structural proteins in epithelial cells (Fuchs, 1995). They are classified according to their biochemical properties (Moll, 1982, Moll, 1992). There are type I or acidic and type II or basic epithelial keratins. Type I keratins are the (cyto-) keratins K9-20 and type II keratins are the (cyto-) keratins K1-8. Furthermore, there are also type I (K31-40) and type II (K81-86) hair keratins (Schweizer, 2006). Other intermediate filaments are vimentin, glial fibrillary acidic protein (GFAP), desmin (which are all Type III intermediate filaments), nestin a type IV, and lamin A a member of type V intermediate filaments.

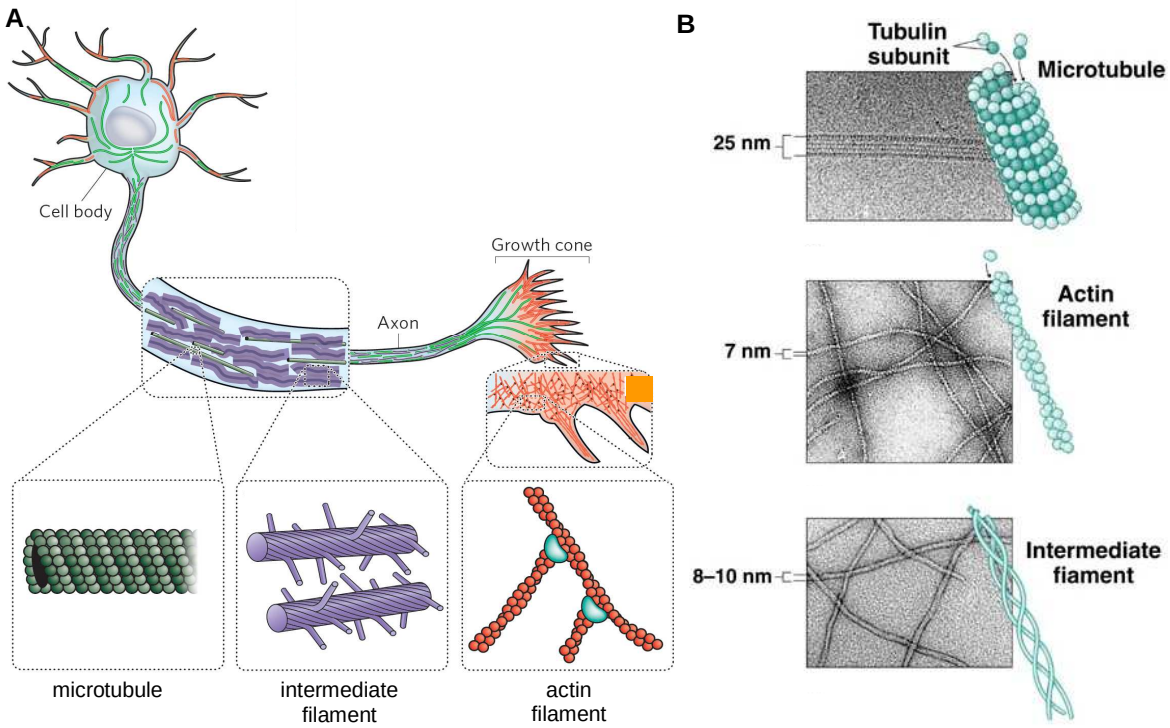

Figure 4: Cytoskeleton of eukaryotic cells. The cytoskeleton supports structure, stress resistance, shape change and movement. (Metzler, 2001).

A) The three different components of the cytoskeleton illustrated in a neuronal cell: microtublues (green), intermediate filaments (purple), actin filament (red).

B) The diameter of the different components is shown in electron-micrographs. (Fletcher, 2010).

The simplest subunit of cytokeratins is a dimer composed of a central α-helical rod domain which is flanked by non-helical amino-terminal head and carboxy-terminal tail domains (Fig. 5 A). This dimer is built from a type I and type II keratin. Type I and II keratins are orientated in parallel in the heterodimer and display a central coiled-coil rod domain (Fuchs, 1998, Coulombe, 2002, Gu, 2007, Arin, 2007). Two of the linear filaments interact in an antiparallel manner, forming the protofilament. In addition, two of the protofilaments form the protofibril. Four protofibrils give rise to the mature intermediate filament, 8-10 nm in diameter (Fig. 5 B). The head and tail domains of the heterodimers are highly conserved ends, so mutations can cause severe consequences in the formation of the intermediate filaments (Letai, 1992).

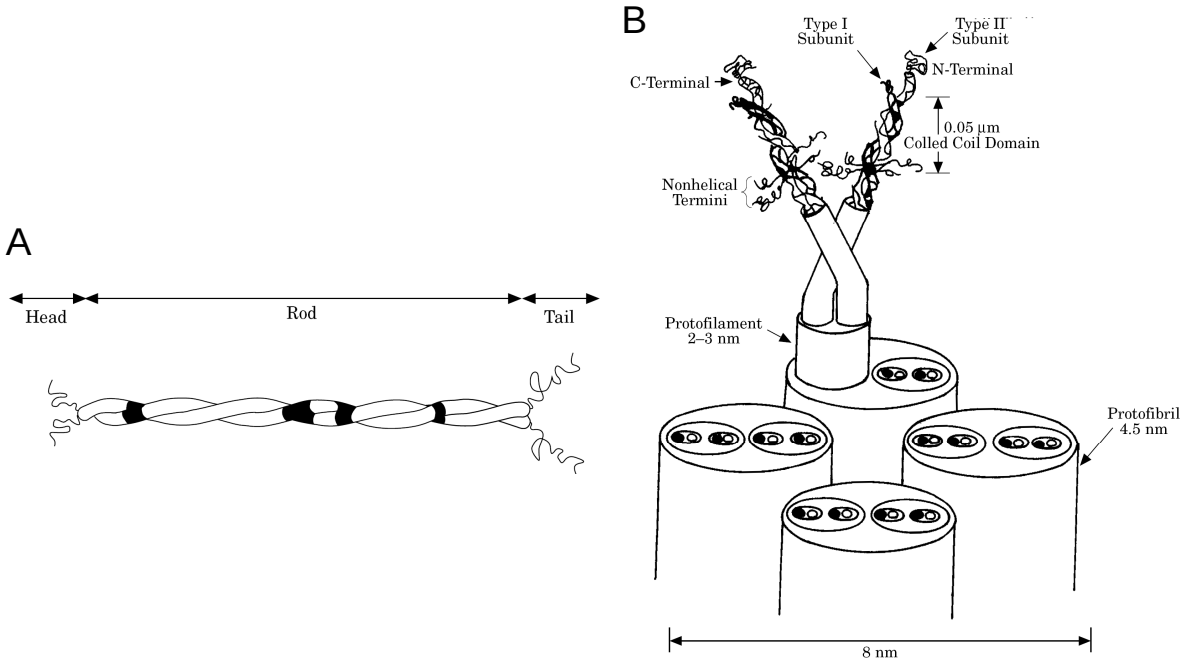

Figure 5: Basic structure and organization of keratin intermediate filaments (IF).
A) The keratin IF dimer structure. The coiled-coil dimer consists of a type I and type II keratin. Hydrophobic interaction of these two α-helical domains results in a central rod domain.
B) Model of the 8-10 nm keratin intermediate filaments. Two dimers are arranged antiparallel forming a protofilament (2-3 nm). The protofibril (4-5 nm) is composed of two protofilaments. Four protofibrils arrange into the intermediate filament which is 8-10 nm in diameter. (Rao, 1996).

Mutations in keratin genes can cause several genetic diseases. Examples for such diseases are the ichtyosis bullosa of Siemens caused by K2 gene mutations (Rothnagel, 1994, Fuchs, 1994, Fuchs, 1997) and K9 gene mutations that result in palmo-plantar keratodermas (Moll, 1982, Vassar, 1991, Reis, 1994, Kuster, 1995, Kuster, 2002). It was shown that epidermolysis bullosa simplex is caused in almost all cases by mutations in the K5 and/or K14 genes (Coulombe, 1991, Bonifas, 1991, Rosenberg, 1991, Lane, 1992).

In the epidermis keratins are indicators of the state of keratinocyte differentiation. In very young skin K15 is expressed in all keratinocytes of the stratum basale, whereas in the skin of older individuals it is expressed in cells of the lower parts of the rete ridges only (Pontiggia, 2009). Suprabasal keratinocytes show no K15 expression (Waseem, 1999; Ghali, 2004; Porter, 2000; Webb, 2004) which implies that in human interfollicular epidermis K15 is not necessarily a stem cell marker (as stated by some authors) but rather a marker for basal keratinocytes anchored to a functional basement membrane (Fig. 6).
K19-positive keratinocytes are highly abundant in the epidermis of fetuses and neonates (Van Muijen, 1987; Kwaspen, 1997). It is still detectable in keratinocytes of the stratum basale of 1.5-year-old children (Pontiggia, 2009). The epidermis of these individuals is characterized by rapid lateral growth. Therefore, it is suggested that K19-positive cells represent a population that is adapted to this rapid lateral expansion of the epidermis and represents a keratinocyte population that is uncommitted to terminal squamous differentiation (Pontiggia, 2009).
Furthermore, cytokeratins K5 and K14 are described to be also expressed in the basal layer of the epidermis (Fuchs, 1980). They are downregulated suprabasally, whereas K1 and K10 are expressed in all epidermal layers except the stratum basale (Moll, 1982, Ishida-Yamamoto, 1998). The expression pattern of keratins is also dependent on the body site. Keratin 9 is only expressed in palmo-plantar skin, K2e is found in spinous and granular layers of interfollicular epidermis (Moll, 1982, Corden, 1996).

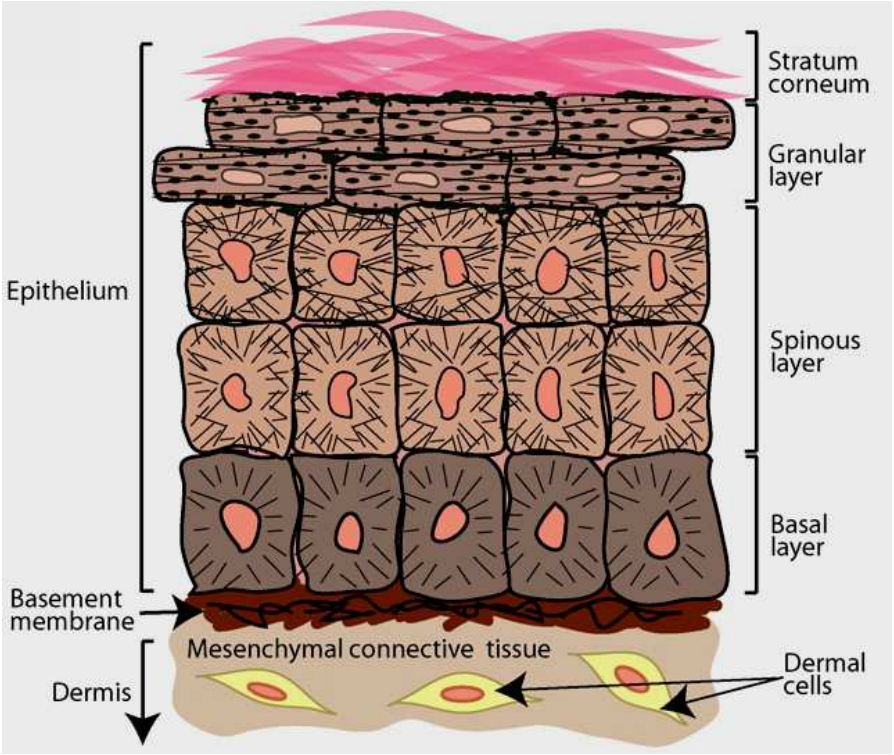

Figure 6: Keratins in human epidermis. Keratins are expressed in different layers of the human epidermis, e.g K15 and K19 in the basal layer, whereas K2e is expressed suprabasally. (Alonso, 2003).

Keratins are important to maintain cell and tissue integrity (Fig. 7 A). They mediate the mechanical support in surface epithelia such as the epidermis, oral mucosa and cornea. Keratins are organized in dense filament networks, linked to cell-cell adhesions like desmosomes and cell-matrix adhesions like hemidesmosomes providing stiffness and strength (Yamada, 2002, Kirfel, 2003, Beil, 2005). Skin blistering can be seen frequently when keratin intermediate filaments get arranged perinuclearly which leads to a softening of the cytoplasm and the detachment of the epidermis from the basement membrane (Beil, 2003).
It was shown that there are also interactions (Fig. 7 B) between intermediate filaments, microfilaments and microtubules (Wiche, 1989, Wiche, 1993, Yang, 1996, Svitkina, 1996, Fuchs, 2001). Such interactions can be mediated through cytolinker proteins like desmoplakin, plectin and bullous pemphigoid antigen (BPAG) resulting in reversibly cross-linked cytoskeletal structures (Leung, 2002).

After injury there is the need for cell migration (Fig. 7 C). Keratinocytes migrate from the wound margins if the epidermis is lost. It was observed that the organization of

intermediate filaments rapidly changes prior to cell migration (Martin, 1997, Pekny, 1999, Omary, 2002, Wong, 2003, Lariviere, 2004). Keratin filaments are rearranged in the perinuclear area to facilitate the migration process (Odland, 1968, Paladini, 1996). Furthermore, a change in keratin expression occurs. Non-wound keratins such as K1/10 are downregulated and wound keratins like K16 are upregulated (Mansbridge, 1987).

Novel functions of intermediate filaments were discovered as it was noticed that they interact with kinases, receptors and adaptors (Fig. 7 D) regulating the cell cycle and programmed cell death (Coulombe, 2000, Omary, 2002, Oshima, 2002, Paramio, 2002, Owens, 2003, Giancotti, 2003, Omary, 2004, Lariviere, 2004). Keratins can modulate the response to pro-apoptotic signalling (Caulin, 2000, Gilbert, 2001, Ku, 2003) through interacting with receptors like Fas receptor or TNFR2 (tumor necrosis factor receptor 2) and regulate cell apoptosis via interaction with TRADD (tumor necrosis factor receptor 1 associated death domain protein) a protein involved in the regulation of apoptosis (Inada, 2001).

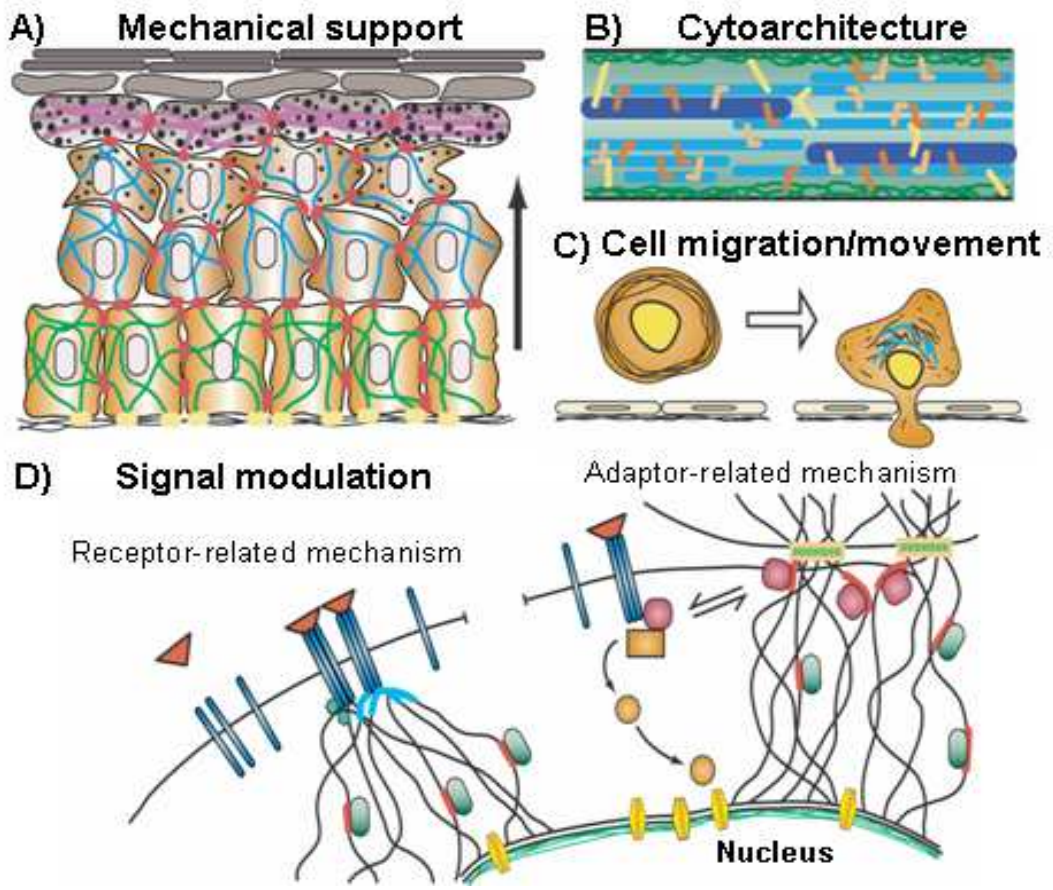

Figure 7: Functions of intermediate filaments.

A) Mechanical support. Keratin intermediate filaments (IF, green in basal layer, blue and pink in suprabasal layers, different colours for different IFs) are abundant in epidermal keratinocytes, in which they are spread throughout the cytoplasm. They are attached to the desmosomes (red dots) and to hemidesmosomes (yellow dots). Therefore, mechanical support in sheets of keratinocytes is maximized.

B) Cytoarchitecture. The architecture of a cell depends also on the interactions of the cytoplasmatic intermediate filaments like keratins (light blue), actin filaments (dark green) and microtubules (dark blue). These interactions are mediated by linker proteins like plectin and bullous pemphigoid antigen BPAG (yellow).

C) Cell migration. Extravasation of a lymphocyte is shown here as an example. Vimentin intermediate filaments are rearranged after activation. Thereby the viscoelastic properties needed for extravasation are changed.

D) Signal modulation. Intermediate filaments can interact with cell surface receptors like Fas (left) modulating their density and function or interaction with adaptor proteins like TRADD (pink on the right) causing limitations of this adaptor to ligand–bound receptor for transmission of a signal. Possibly these mechanisms are involved in the response of epithelial cells to pro-apoptotic or other signals. BPAG: Bullous Pemphigoid Antigen, IF: intermediate filaments, TRADD: TNFRSF1A-associated via death domain. (Coulombe, 2004).

Moreover, keratins are implicated in the control of protein synthesis and cell proliferation. Recently, it was shown that K17 binds 14-3-3σ, an adaptor protein regulating mTOR (mammalian target of rapamycin) activity. The K17/14-3-3σ interaction influences protein synthesis and cell proliferation (Bertram, 1998, Kim, 2000, Bridges, 2005). Keratin 10 is suggested to interact with the kinase Akt to influence proliferation *in vivo*. Overexpression of K10 in cell lines and mouse models reduces cell proliferation (Paramio, 1999, Santos, 2002) supporting the thesis that K10 expressed in suprabasal layers in normal epidermal keratinocytes triggers differentiation (Paramio, 2002).

Keratins also play a role in the pigmentation of skin. The mechanisms still remain vague. It is known that several disorders, e.g. the Naegeli-Franschetti-Jadassohn syndrome, caused by a K14 mutation, displays (as one of several symptoms) a reticulate or mottled hyperpigmentation (Lugassy, 2006). Hyperpigmentation and dark hyperkeratotic papules are seen in the Dowling-Degos-disease, a disorder linked to keratin 5 haploinsufficiency (Betz, 2006).

Several authors suggest that keratins are also markers for epithelial stem cells. Keratin 15 and 19 are postulated candidates as stem cell markers (Lane, 1991, Jones, 1995, Michel, 1996, Lyle, 1998). Some hints point toward this suggestion but the final proof for this hypothesis is still missing.

1.2.2.2 Cornification of human epidermis

Cornification of the epidermis provides a barrier against water and physical resistance to mechanical forces (Michel, 1988, Kalinin, 2002, Presland, 2002, Segre, 2003). Cornification actually starts in the granular layer. There, the keratinocytes synthesize immature structural proteins that are stored beneath the plasma membrane. Later a rigid structure is formed by covalent attachment of linker proteins, such as involucrin and loricrin (Fig. 8). The differentiated keratinocytes also acquire so-called keratohyalin granules containing profilaggrin. Keratin filaments are aggregated via mature filaggrin into tight bundles which are closely aligned in regular arrangements (Steinert, 1981). After this aggregation the intermediate filaments “collapse”, leading to a flattening of the former ellipsoid cells (transforming into flat squames) which is characteristic for cells in the cornified layer.

Structural proteins, such as glutamine and lysine-rich proteins involucrin and loricrin, and small proline-rich proteins (SPRs), are cross-linked to tight keratin bundles beneath the plasma membrane through transglutaminases (Fig. 8). This process reinforces the formation of tight bundles forming the cornified envelope. Involucrin and loricrin are the main components of the cornified envelope, constituting 70-80 % of the protein mass in the cornified layer (Steinert, 1995, Steinert, 1998, Kalinin, 2001, Candi, 2005). Desmosomes are also modified during keratinocyte differentiation. In the stratum spinosum they are mediating the tight attachment of keratinocytes, whereas in the upper strata they are proteolytically degraded to allow desquamation (Serre, 1991).

Not only proteins but also lipids are involved to establish the cornified layer. Lipids are attached to the proteins of the cornified envelope, or they arrange into intercellular lamellae to support the water barrier function.

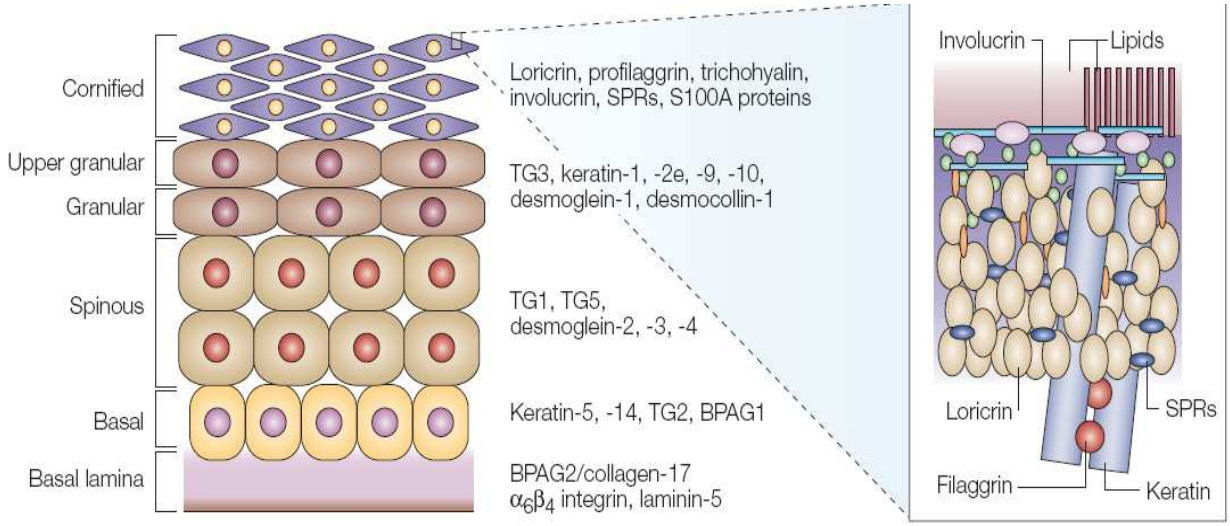

Figure 8: Cornification in human epidermis.

Starting in suprabasal layers, several proteins are expressed in particular regions in the epidermis during epidermal differentiation. The cornified envelope is established by crosslinking of keratin with proteins like loricrin or involucrin by transglutaminases (inset on the right). (BPAG, bullosus pemphigoid antigen; SPR, small proline-rich proteins; TG, transglutaminase). (Candi, 2005).

1.2.3 Human melanocytes

Neural crest derived melanocytes migrate during embryogenesis via the mesenchmye into the epidermis and hair follicles. Interestingly, melanocytes also migrate to the cochlea in the inner ear, to the leptomengines and to the uveal tract of the eye. In the hair follicle melanocytes are situated in the outer root sheath and in the hair matrix above the dermal papilla where they are responsible for hair color (Bolognia, 2003, van Neste, 2004, Slominski, 2005). It is hypothesized that the hair follicle melanocyte stem cells are found in the hair follicle bulge, whereas the interfollicular epidermis contains its own melanocyte stem cells in the basal layer (Tobin, 1996, Passeron, 2007, Tobin, 2008, Blanpain, 2010). In the basal layer of the epidermis (Fig. 9) the ratio of melanocytes to keratinocytes is about 1:5 (Haass, 2005).

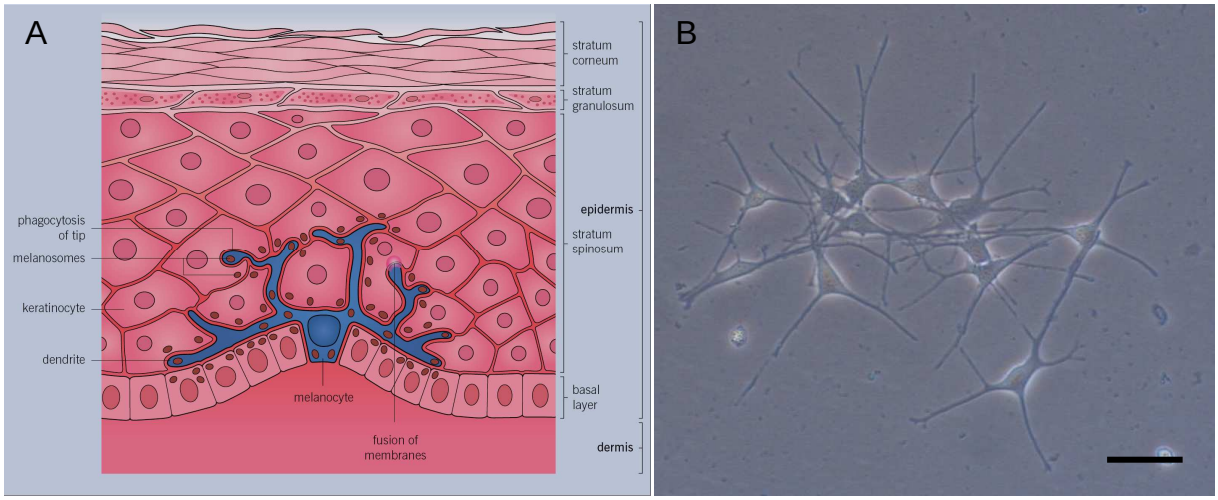

Figure 9: The human epidermis contains melanocytes.
A) The basal layer of human epidermis contains melanocytes. With its dendrites one melanocyte is in contatct with about 36 keratinocytes located in different layers of the epidermis. Melanosomes containing the pigment melanin are transferred along the dendrites to keratinocytes. (Bolognia, 2003).
B) Human melanocytes (here displaying their dendritic processes on cell culture plastic) were isolated in our laboratory from human skin biopsies. They were expanded as a homogeneous and pure cell population in a specific melanocyte medium. (bar, 50 µm).

The so-called epidermal melanin unit describes the number of keratinocytes being provided with melanin by one melanocyte. One melanocyte is associated with about

36 keratinocytes in the epidermis (Fitzpatrick, 1963, Jimbow, 1976). Melanin is important to protect the skin against ultraviolet (UV) radiation via its optical and chemical filtering properties (Ahene, 1995). Melanosomes containing the pigment melanin are translocated via a microtubule and actin filament driven process to the tips of the dendritic protrusions of the melanocytes (Jimbow, 1998, Lambert, 1999, Wu, 2000, Wasmeier, 2008).

The transfer process of melanosomes from melanocytes to keratinocytes is not entirely understood. Several hypotheses are suggested for this process such as release of melanosomes from melanocytes into the intercellular space subsequently followed by endocytosis by keratinocytes or keratinocyte-membrane fusion and uptake of melanosomes by keratinocytes through the “fusion channel” (Yamamoto, 1994, Jimbow, 1998, Boissy, 2003). Apparently, the PAR-2 (protease activated receptor 2) receptor plays a crucial role in the uptake of melanosomes (Sharlow, 2000, Seiberg, 2000, Scott, 2001, Cardinali, 2005). Once taken up into the keratinocytes, melanosomes are situated in supranuclear caps (Fig. 10).

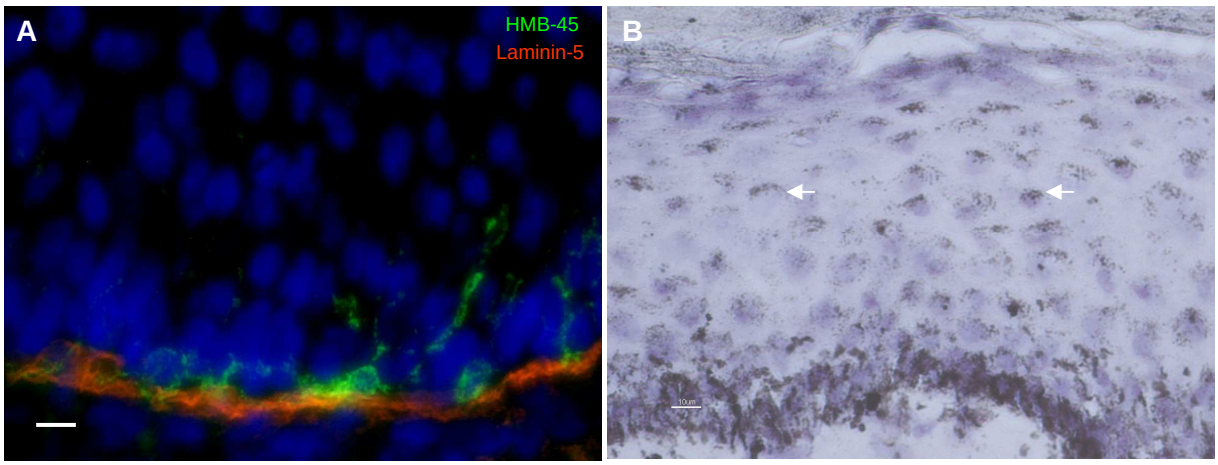

Figure 10: Human dermo-epidermal skin substitutes containing melanocytes.

A) Human melanocytes (green) reside in our dermo-epidermal skin constructs in the basal layer of the epidermis which always is in contact with the basement membrane (Laminin-5, red). The antibody HMB-45 (green) detects melanosomes in the melanocytes. Therefore, the dentritic protrutions towards the upper layers of keratinocytes in the epidermis are also stained.

B) Fontana-Masson staining reveals melanin (brownish-black dots) in the epidermis in our substitutes. White arrows denote melanin, located in supranuclear caps (black grains) in the keratinocytes. (Biedermann, manuscript in preparation). This is in accordance with the normal in vivo situation of human skin. (bars, 10 µm).

Melanin can be divided into two main types, pheomelanin (red/yellow) and eumelanin (black/brown). The pigments, pheomelanin and eumelanin, are both synthesized and stored in melanosomes. Several morphologically distinguishable stages (I-IV) are observed in the complex process of melanosome biogenesis (Fig. 11).

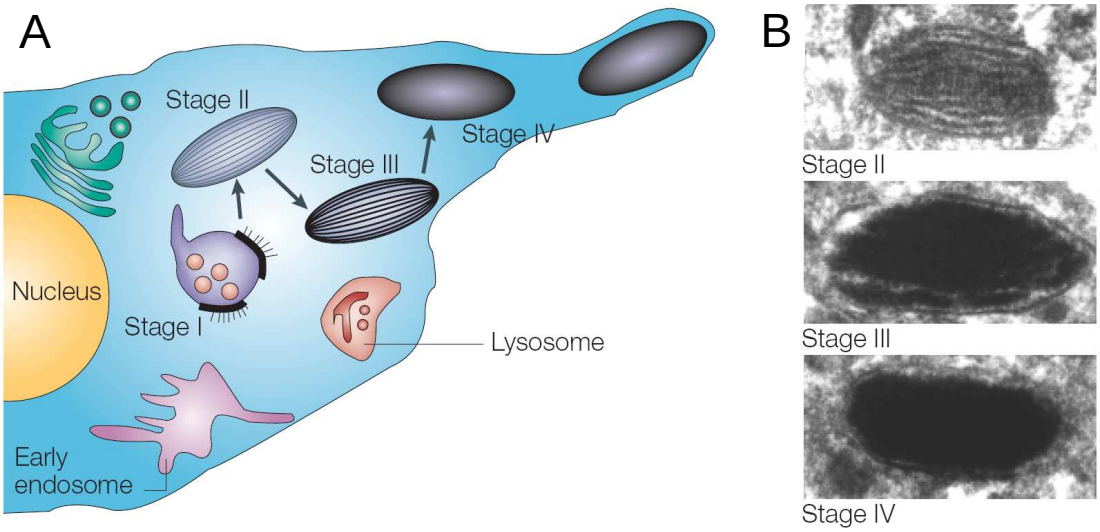

Figure 11: Maturation of melanosomes.
A) Schematic representation of dendritic process of a melanocyte. There are different stages of maturation from I to IV. The intensity of black color indicates the degree of melanization.
B) Transmission electron microscopy pictures displaying stage II to IV melanosomes in human melanocytes. (Marks, 2001).

Both pigments accumulate in the organelles by enzymatic oxidation of tyrosine, later the pathways for pheomelanin and eumelanin separate (Raposo, 2002, Kushimoto, 2003, Setaluri, 2003). Eumelanin undergoes further hydroxylation, oxidation and carboxylation processes. Pheomelanin needs at least one reduction reaction which is cysteine dependent (Hearing, 1999). Genetic disorders affecting the melanosomes display defects in skin and hair pigmentation e.g. oculo-cutaneous albinism in humans (Oetting, 2003).

1.3 Skin appendages

1.3.1 Hair follicles

Hair follicles are multilayered cylindrical skin appendages (Fig. 12) formed mainly by cells of epidermal origin.

A crosstalk between keratinocytes and dermal cells is required in fetal skin to establish the hair anlagen. These signaling cues include Wnt/β-catenin, sonic hedgehog (Shh), fibroblast growth factors (FGFs), and bone morphogenetic proteins (BMPs) (Fuchs, 2008). Several lines of evidence suggest that activation of Wnt/β-catenin signaling in epithelial cells is a key initial step in placode formation (Holbrook, 1991, Hardy, 1992, Botchkarev, 1999, Milliar, 2002, Botchkarev, 2002, Ito, 2007). The epidermal cells differentiate, followed by the induction of dermal cells to form a condensed defined area of mesenchymal cells. In turn, the dermal cells express proteases cleaving the extracellular matrix to pave the way for the ingrowth of the originating hair follicles into the dermis (Karelina, 1993, Karelina, 1994, Karelina, 2000).

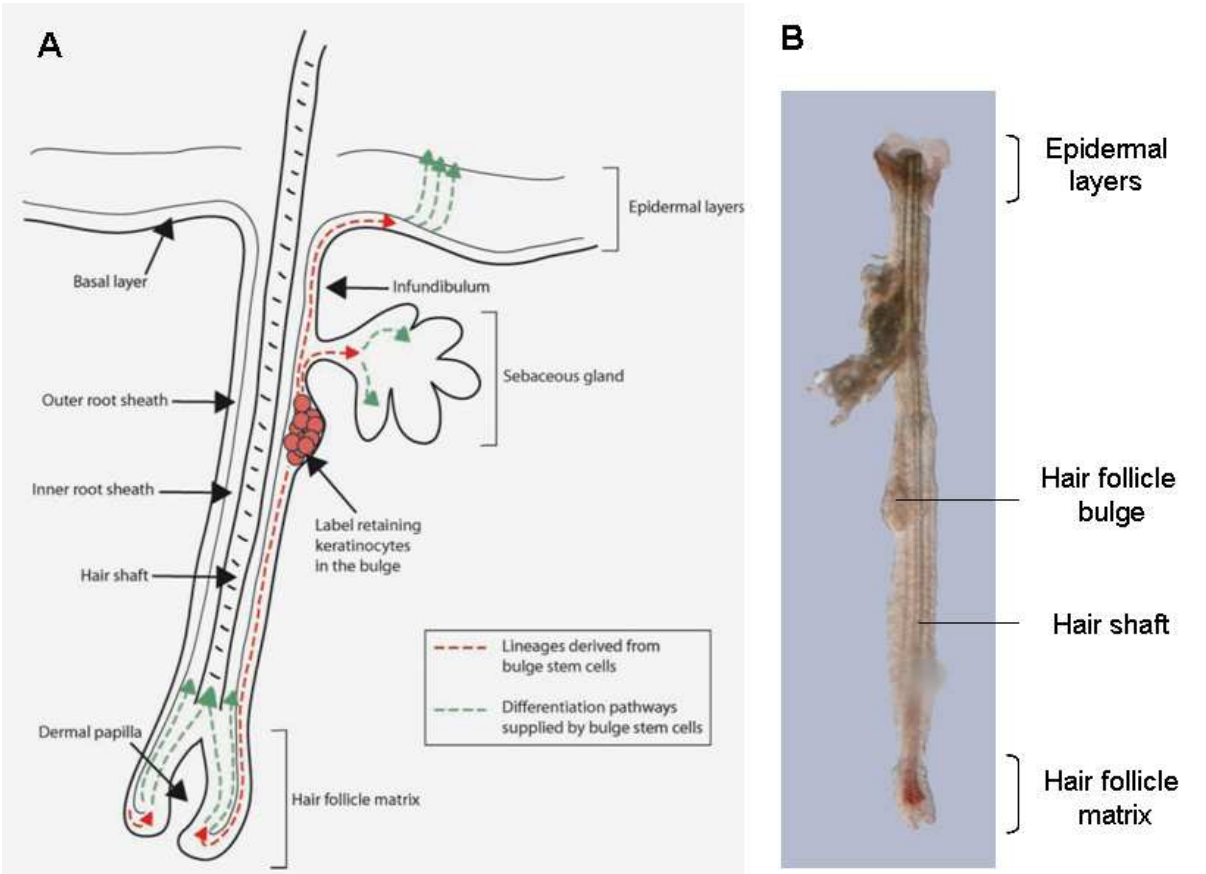

Figure 12: The hair follicle in a schematic overview and an isolated human hair follicle.
A) The pilosebaceous unit containing the hair follicle and the sebaceous gland are embedded in the dermis. The outer root sheath forms the external sheath of the follicle. The inner root sheath forms the channel for the hair itself. The bulge, a specialized compartment in the outer root sheath below the sebaceous gland, contains multipotent stem cells. These cells can give rise to cells of the hair follicle, the sebaceous gland and in a wound situation to cells of the epidermis. Mesenchymal cells in the dermal papilla interact with hair matrix cells necessary to regulate the hair cycle. (Alonso, 2003).
B) Complete human hair follicle isolated in our laboratory from human scalp donor tissue.

Hair growth is a tightly regulated regeneration cycle classified into three phases:
1. anagen (phase of active hair growth),
2. catagen (follicle regression)
3. telogen (follicle rest) (Hardy, 1992, Stenn, 2001).

Dermal papilla cells secrete growth factors and other signaling molecules that regulate proliferation and differentiation of keratinocytes during the hair cycle

(Messenger, 1991a, Messenger, 1991b, Shimaoka, 1994, Rudman, 1997, Paus, 1999, Kulessa, 2000, Botchkarev, 2003).

In the anagen phase the lower portion of the follicle is generated. Keratinocytes in the so-called bulb, which is located above the dermal papilla, proliferate and give rise to inner root sheath cells and hair shaft cells. The catagen period is typified by apoptotic cell death of the lower two-thirds of the hair follicle (Weedon, 1981). As the hair follicle regresses, the dermal papilla cells maintain their contact with the basement membrane. This draws the dermal papilla up to the base of the permanent epithelial portion of the follicle.

At this point, the follicle enters a resting phase known as telogen. The telogen phase can last for months in humans. The old non-anchored hair shaft becomes fragile and can be liberated from its site when physically stressed (Fuchs, 2001).

After this resting period, a new hair cycle is initiated by a hair germ-like structure.

The outer root sheath harbours a specialized region, the bulge. This part of the hair follicle is located below the sebaceous gland at the insertion site of the arrector pili muscle (Stohr, 1903, Pinkus, 1958, Cotsarelis, 1990). In contrast to rodents, the bulge of human adults is less distinctive (Rochat, 1994, Cotsarelis, 2006). The microenvironment of the bulge enables hair follicle stem cells to be maintained in this specialized niche region (Cotsarelis, 1990). The self-renewing bulge stem cells exit the niche in the anagen phase, proliferate and differentiate into several cell lineages of the hair follicle (Morris, 1994, Morris, 1999, Oshima, 2001, Braun, 2003, Morris, 2004, Ohyama, 2006). In addition, these stem cells are also recruited in wound healing, giving rise to keratinocytes to repair the epidermal defect (Wilson, 1994, Ghazizadeh, 2001, Oshima, 2001, Tumbar, 2004, Blanpain, 2004, Levy, 2007, Langton, 2008). Keratinocyte stem cells located in the bulge are not involved in homeostatic maintenance of the epidermis (that is in non-wound situations) as it was postulated (Taylor, 2000). It was convincingly shown in several studies that the epidermis contains unipotent progenitor cells able to maintain tissue homeostasis (Potten, 1988, Lavker, 2000, Morris, 2004, Ito, 2005, Levy, 2005). Furthermore, palmo-plantar skin lacks hair follicles, but normal epidermal homeostasis and wound healing are observed (Montagna, 1954), indicating that hair follicles are not an indispensable source of keratinocyte stem cells.

1.3.2 Eccrine and apocrine sweat glands

About 2 to 4 million sweat glands are dispersed over the human body (Saga, 2002, Groscurth, 2002). Sweat glands are classified according to morphology and function as eccrine and apocrine sweat glands.
The highest density of eccrine sweat glands can be found on palms and soles, the axillae or the forehead. Sweat glands are absent in lips, nail beds, the inner preputial surface or the glans penis (Szabo, 1967, Sato, 1977).

Eccrine sweat glands are important in the thermoregulation of humans and primates. The body temperature is lowered by evaporation of sweat. Sweat glands can produce up to 10 liters of sweat per day. The excreted sweat is an odorless, clear and hypotonic fluid. It contains sodium, potassium, chloride, calcium, amino acids, lactate, urea and immunoglobulins (Shibasaki, 2010). Patients lacking sweat glands suffer from increased body temperature in hot environments or during physical activities. Such patients are burn victims with deep large-area burns or patients with congenital anhidrotic ectodermal dysplasia (Christ-Siemens-Touraine syndrome), the latter characterized by abnormal or absent sweat glands, teeth, nails and hairs (Blecher 1986, Loth, 1998, Priolo, 2000, Gaide, 2003, Drögemüller, 2003, Itin, 2004).

The development of eccrine sweat glands is detectable during the third month of embryogenesis (Hashimoto, 1965, Sato, 1989 Moll, 1992, Ersch, 1999). Epithelial downgrowth from epidermal ridges can be seen and solid cords elongate into the dermis. The secretory domain of the gland is formed by the lower end of the ductal cord in the dermis. The fusion of intracytoplasmatic vacuoles and the detachment of desmosomes lead to the formation of a lumen between the fourth and eighth month of pregnancy. Although they display no function before birth, mature sweat glands are noticeable at about week 32.

Apocrine sweat glands are located in areas such as the axilla, the perianal region, the scrotum and the prepuce. These glands produce a thick, milky, odorless fluid rich in proteins, ammonia, lipids and carbohydrates. A characteristic smell is produced when bacteria residing on the skin decompose the produced fluid (Groscurth, 2002).

Eccrine sweat gland consists of two main segments, a duct and a secretory domain (Fig. 13). The excretory duct extends through the dermis and opens to the epidermis (Hashimoto, 1966, Ellis, 1967, Montagna, 1974, Hurley, 2001, Langbein, 2008). It is composed of two cell layers, a peripheral cell layer and an inner cell layer. The duct is segemented in a proximal coiled intraglandular duct, a straight intradermal duct and an intraepidermal spiral duct, known as the acrosyringium (Langbein, 2005).

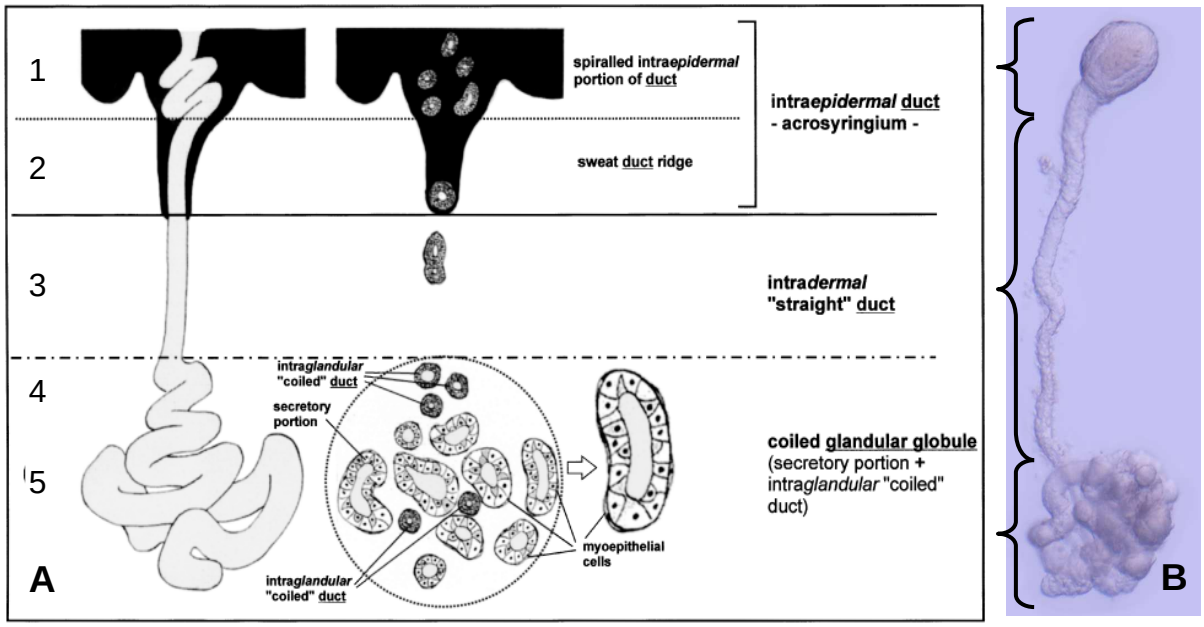

Figure 13: The human sweat gland.

A) The eccrine sweat gland and its main segments are shown in a schematic presentation (Langbein, 2005). The numbers on the left indicate the segments:

(1) upper spiral intraepidermal duct of the acrosyringium

(2) lower sweat duct ridge of the acrosyringium

(3) intradermal duct

(4) intraglandular duct portion

(5) secretory duct portion.

B) This complete gland was isolated in our laboratory for subsequent cell isolation. The parentheses denote the different segments according to the schematic presentation.

A basal (outer) and a luminal (inner) cell layer form the duct. Luminal cells, exhibiting a Na-K-ATPase activity, reabsorb solutes like sodium chloride and lactate from the isotonic fluid produced by the secretory domain (Bijman, 1987, Reddy, 1989, Reddy, 1991, Reddy, 1994, Reddy, 1999, Granger, 2003).

The coiled single layer secretory domain is composed of myoepithelial cells, dark and clear secretory cells. Clear cells have a high transepithelial activity in fluid and electrolyte transport. The majority of the sweat, water and electrolytes, is secreted by these cells. The function of dark cells is not known, although it was observed that they secrete PAS-positive glycoproteins into the sweat (Sato, 1993).

The spindle shaped myoepithelial cells express cytokeratins and also α-smooth-muscle actin. By their contraction the sweat is “pumped” through the duct. In addition, myoepithelial cells provide mechanical strength and serve as a protection against overexpansion in large volume sweat secretion phases (Sato, 1979, Sato, 1980).

Eccrine sweat gland cells serving as a stem cell resource

In contrast to hair follicles, sweat glands are not in focus of research in respect to molecular signalling, development or maintenance throughout life. Of course, this lack of knowledge can be explained by the absence of an appropriate animal model, to study the biology of sweat glands.

In analogy to the stem cell containing bulge in hair follicles, it is proposed that sweat glands harbour (keratinocyte) stem cells (Barrandon 2003, Gambardella 2003, Nakamura, 2009). Although data are rare, it is known that the acrosyringium is a non-self-renewing segment (Lobitz, 1954, Christophers, 1973, Kanitakis, 1987). Hence, the intraepidermal duct cells are renewed by upward migration of intradermal duct cells. It was hypothesized that under homeostatic conditions luminal cells at the base of the intraglandular duct are able to maintain the integrity of the luminal duct layer (Langbein, 2005). Other authors described transitional cells, ranging in phenotype between myoepithelial cells and dark and clear cells, suggesting a pool of stem cells in the secretory portion (Sato, 1993).

1.4 The Dermis

1.4.1 Structure and function of human dermis

The dermis is a connective tissue underlying the epidermis. It harbours the epithelial appendages, provides mechanical support and is important for epithelio-mesenchymal interactions. The dermis is rich in extracellular matrix, such as collagens, elastin and glycosaminoglycans. The papillary dermis displays a higher number of fibroblasts and has a different tissue organisation compared to the reticular dermis (Fig. 14). Beside this, a lot of microvascular structures pass through the papillary dermis, especially the dermal papillae, to assure blood supply for nourishment of the epidermis (Cormack, 1987). The rete subpapillare denotes the border between papillary and reticular dermis. The reticular dermis extends to the rete cutaneum, the boundary to the adipocyte rich hypodermis. Skin appendages often reach through the reticular dermis to terminate in the hypodermis (Sorrell, 2004, Mine, 2008).

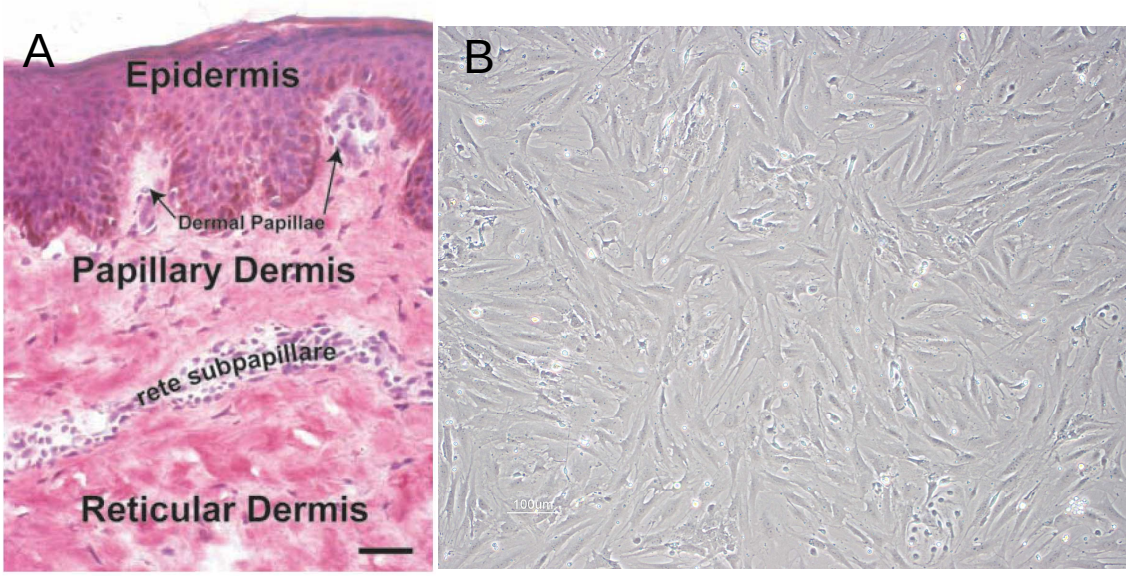

Figure 14: Histological section of human skin and isolated human dermal fibroblasts.
A) The papillary dermis, with dermal papillae extending the contact surface of epidermal-mesenchymal ridge, is the dermal part underlying the epidermis. This part of the dermis contains a higher cell density compared to the reticular dermis. The reticular dermis is separated by the rete subpapillare from the papillary dermis (bar, 45 µm). (Sorrel, 2004).
B) Human dermal fibroblasts isolated from a skin biopsy in cell culture (bar, 100 µm).

1.4.2 Basement membrane deposition and scar formation – two functions of dermal fibroblasts

Fibroblasts are important for the biosynthesis of extracellular matrix components, cytokines, growth factors and basement membrane components (Smith, 1997, Postlethwaite, 1999). Moreover, they remodel the connective tissue by expression of matrix metalloproteinases (MMPs) (Page-McCaw, 2007).

Both, fibroblasts and keratinocytes, are involved in the production of basement membrane components. The basement membrane is a multi-molecular structure (Fig. 15) on which the basal epithelial cells reside. It separates the epidermis physically from the underlying dermis, whereas it connects them functionally (Burgeson, 1997, Aumailley, 1999, LeBleu, 2007).

Dermal fibroblasts express laminin-1, nidogen, collagen type IV and VII. Epidermal keratinocytes produce laminin-5, perlecan and collagen IV and VII (Contard, 1993, Marinkovich, 1993, Fleischmajer, 1995, Moulin, 1995, Smola, 1998). A cooperative effort of both epidermal keratinocytes and dermal fibroblasts is necessary to form a basement membrane (Fleischmajer, 1993, Moulin, 2000, Sorrell, 2004). One example of this interaction is the fact that transforming growth factor-β1 (TGF-β1) secreted by dermal fibroblasts activates the expression of collagen VII by epidermal keratinocytes (König, 1991, König, 1994, Monical, 1994, Fusenig, 1994).

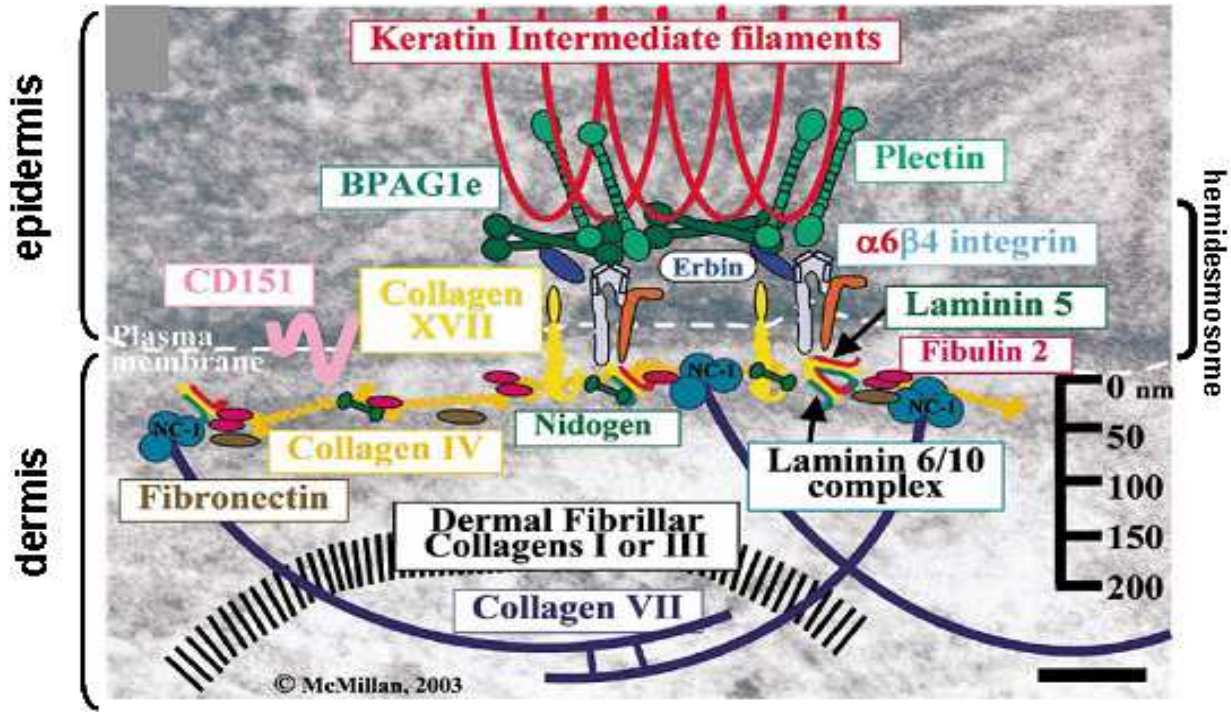

Figure 15: Schematic diagram depicting the relative positions of selected epidermal basement membrane components.

The diagram demonstrates the series of structural proteins that form a continuous link from keratin intermediate filaments to dermal collagens via the hemidesmosome complex. Keratin intermediate filaments (red) within the keratinocyte cytoplasm are capable of binding both plectin (light green rods) and bullous pemphigoid antigen 1 (dark green rods, BPAG1e). These two plakin family protein members form a series of interactions (via erbin, in blue) with the two major hemidesmosome transmembrane molecules, integrin α6β4 (red and blue) and collagen XVII (yellow). Integrin α6β4 is the receptor for the extracellular ligand laminin-5 (red, yellow and green). Laminin-5 in turn binds to the collagen VII NC-1 domain (dark blue circles), to nidogen (small green dumbbells), fibronectin (brown oval) and fibulin 2 (pink oval). The majority of collagen VII molecules form semicircular loop structures (dark blue loops) called anchoring fibrils (collagen VII) immediately beneath the lamina densa. In the dermis there are dermal collagen fibres comprising collagens type I and III (parallel black lines) (Scale bar: 100 nm). (McMillan, 2003).

Dermal fibroblasts organize and maintain the connective tissue in homeostasis. Moreover, fibroblasts play a crucial role in response to injury (Shaw, 2009).

Skin wound healing involves two main phenomena (Tomasek, 2002). On the one hand, it means re-epithelialization, which involves proliferation and migration of keratinocytes to reconstitute the epidermis.

On the other hand, de novo formation and contraction of dermal granulation tissue appears. The granulation tissue is essentially composed of small vessels, fibroblasts, myofibroblasts and inflammatory cells (Martin, 1997). Contraction of the wound is caused, at least in part, by the presence of myofibroblasts which develop characteristics of smooth muscle cells (expression of α-smooth-muscle actin and non-muscle myosin) under the influence of TGFβs (Kreis, 1980, Singer, 1984, Skalli, 1986, Darby, 1990, Burridge, 1996, Chicurel, 1998, Dugina, 2001, Geiger, 2001). In contrast to embryonic wound healing (Adzick, 1994, Gurtner, 2008), which still occurs without scarring, wound contraction is the general mode of wound healing in humans after birth (Liechty, 2000, Harrison, 2008).

Fibroblasts produce at fist a provisional wound matrix containing hyaluronan and fibronectin. Secondly, they remodel the granulation tissue by formation of a collagenous matrix (Clark, 1990, Gailit, 1994, Juhlin, 1997, Singer, 1999, Wong, 2007). However, the new (scar) tissue will have a distinct collagen pattern and a different skin texture (and quality) than the initial (and surrounding) normal skin.

1.5 Tissue Engineering

1.5.1 Tissue engineering – defininition, achievements, state of the art

In 1987 the Washington National Science Foundation bioengineering panel adopted the term “tissue engineering” (Nerem, 1992). This term stands for the “application of the principle methods of engineering toward the development of biological substitutes” (Atala, 2007). There is the need for organ transplantation in patients suffering from life-threatening diseases. It is possible to transplant organs like hearts, livers, kidneys or lungs from organ donors, but of course the need exceeds the supply. Organ transplantation from other species (especially from pigs), so-called xenotransplantations (Hecht, 2009), remain speculative not only because of the immunological barrier or ethical problems but also because of microbiological hazards (like pig endogenous retro-virus). Tissue engineering overcomes such problems like immunological rejections, as autologous cells from the patient are used.

Today, there is a broad spectrum of possibilities in the field of tissue engineering. Worldwide there is research and engineering for almost every organ or tissue, the outcome of which remains sometimes suspect. Nevertheless, successfully applied approaches in clinical use display that there is progress in tissue engineering. Some examples of progress in tissue engineering are shown in Fig. 16. Other fields of tissue engineering not shown in Fig. 16 include the liver (Kaufmann, 1999, Kim, 1999), the lung and the trachea (Kojima, 2003, Kanzaki, 2006, Macchiarini, 2008), the kidney (Steer, 2004, Atala, 2007), the salivary gland (Baum, 1999, Aframian, 2007, David, 2008) and esophageal cell sheet transplantation (Ohki, 2006, Murakami, 2006).

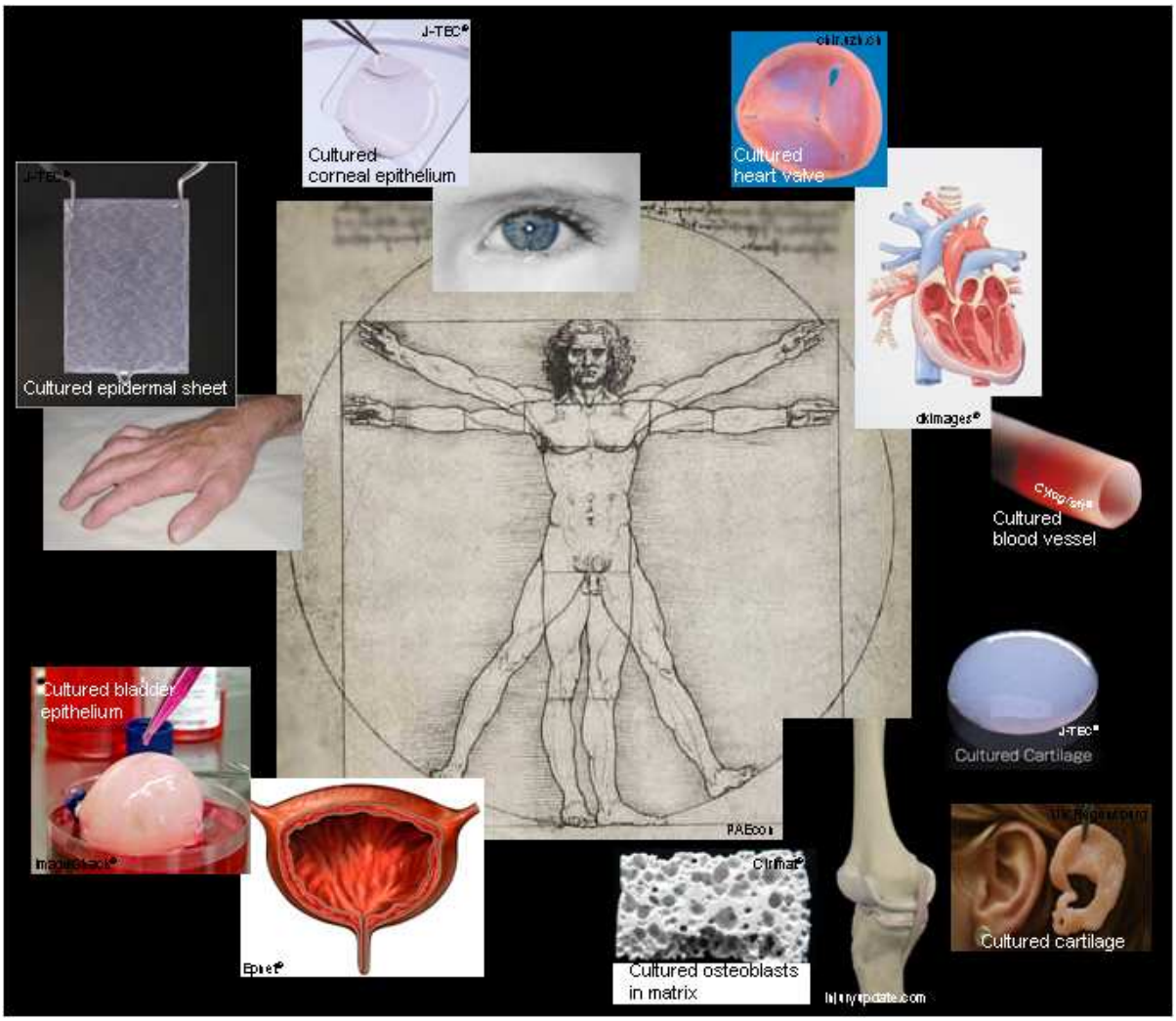

Figure 16: Current state of human tissue engineering.

Examples of research areas in the field of tissue engineering include bladder (Oberpenning, 1998), epidermal/corneal cell sheet (Ronfard, 2000, Nishida, 2004, Hata, 2007), heart valve (Shinoka, 1995, Stock, 2000, Schmidt, 2007), blood vessels (Levenberg, 2005; L'Heureux, 2007), cartilage (Chung, 2008), intervertebral disc (Mizuno, 2005), ear (Cao, 1997) and bone (Yoshikawa, 2000). Note that in several areas of research different achievements were obtained. In some fields clinical trials are undertaken.

1.5.2 Tissue Engineering of skin

There is a great need for tissue engineered skin because the loss of skin can occur by many diseases and accidents. The western world faces increasing life expectancy, hence one of the most common skin defects are chronic, non-healing ulcers in elderly people (Fig. 17 A) (Falanga, 1993, Paquette, 2002, Falanga, 2005, Dalla Paola, 2006, Gary Sibbald, 2008). There is also a need for skin to treat congenital disorders such as giant melanocytic (melanocyte) naevi (Fig. 17 B) or epidermolysis bullosa, where large areas need to be replaced by healthy skin (Zaal, 2004, Bauer, 2005, Earle, 2005, Tannous, 2005, Aumailley, 2006, Mavilio, 2006, Arneja, 2007, Fine, 2008). Furthermore, in reconstructive surgery treating burn accidents followed by scar management (Fig. 17 C), tissue engineered skin could be favourably used (Swope, 2002, Atiyeh, 2005, Harrsion, 2008). In addition, studying wound repair and cancer biology have a need for laboratory grown skin (Garlick, 2007, Andreadis, 2007, Carlson, 2008).

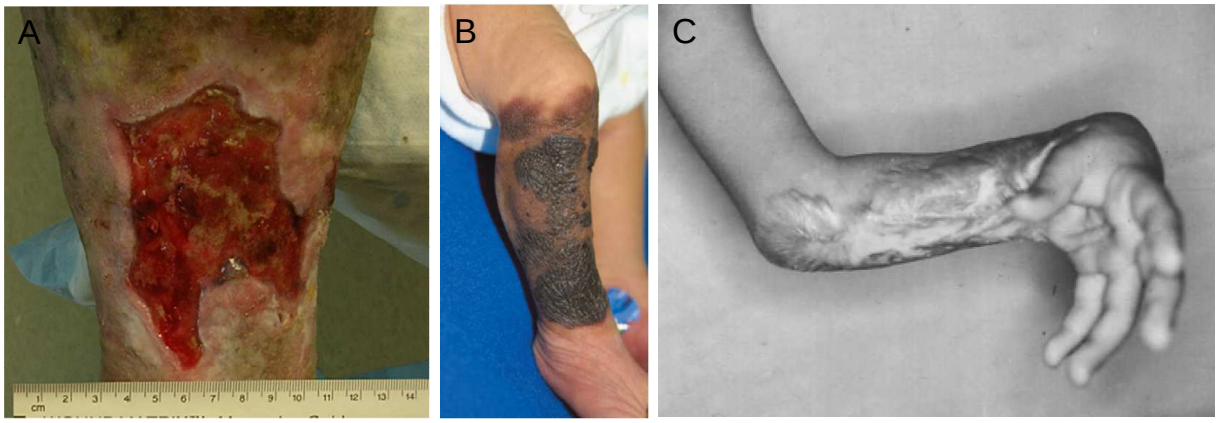

Figure 17: Different examples demonstrating the need for tissue engineerd skin.
A) An adult patient suffering from venous stasis leg ulcer, a common chronic wound. (Fonder, 2008).
B) Congenital giant nevus of the leg of an infant. (Kryger, 2008).
C) Scar contracture on the wrist after a burn injury. (Tomasek, 2002).

The today's gold standard to cover large wounds is the transplantation of autologous split-thickness skin (Pereira, 2007). Split-thickness skin contains all of the epidermis but only partially dermis (Fig. 18 B), and if transplanted without good dermal support, frequently leads to scarring (Arabi, 2007, Barbour, 2008, Berman, 2008).

In contrast, full-thickness skin transplantation (Fig. 18 C) is not usually associated with scarring, however autologous full-thickness skin transfer can only be performed for injured areas <2% total body surface area (TBSA) (Rivera, 2005).

Concerning the application of laboratory grown skin, a major breakthrough was made in 1975, when Rheinwald and Green managed to grow human primary epidermal cells in serial culture on a layer of lethally irradiated 3T3 murine fibroblasts (Rheinwald, 1975). These researchers showed that limitations observed previously in the cultivation of epidermal cells in surface cultures were not intrinsic, but due to the complex relationship between keratinocytes and fibroblasts. Taking advantage of the 3T3 cell feeder layer technique, an epidermal graft could be expanded to more than 500 times its size within 3–4 weeks (Rheinwald, 1977). After the first clinical application (O´Connor, 1981), so-called cultured epidermal autografts (CEAs) were tested in almost all leading burn centers world wide (Fig. 18 A) (Gallico, 1984, Odessey, 1992, Atiyeh, 2007, Guerra, 2009). However, there were disadvantages that included a widely variable CEA take, depending on the wound site and status, age of the patient, and knowledge and experience of the operator (Munster, 1996, Wood, 2006).

Often, an acellular dermal template, such as Integra Artificial Skin, was applied in a first surgical intervention to yield a well-vascularized dermal wound bed onto which split-thickness skin was transplanted in a second surgical step (Ruszczak, 2003, Horch, 2005, Hata, 2007, MacNeil, 2007, van der Veen, 2010).

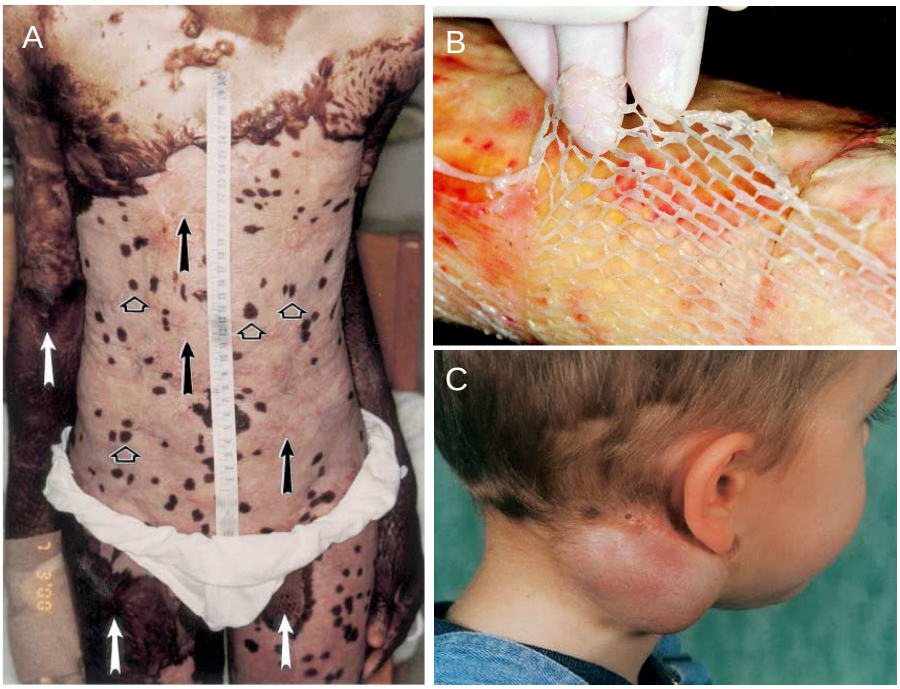

Figure 18: Examples for skin grafts.

A) A black, 11-year old burn patient was treated with cultured epidermal autografts (CEAs) on the abdomen and anterior thighs. Nine months after grafting hypopigmentation was observed (black arrows) in the grafted areas, whereas there was normal pigmentation in areas in which full-thickness meshed autografts were transplanted (white arrows). Contamination with autologous melanocytes caused hyperpigmented spots in the transplanted keratinocyte cell sheets (open arrowheads). (Swope, 2002).

B) Example of an autologous split-thickness skin mesh. The meshed skin was transplanted onto an Integra-based neodermis. (Loss, 2000).

C) Example of application of a tissue expander for full-thickness skin transplantation. A 3 year old boy displaying a pigmented naevus retroauricular. A self-filling tissue expander was implanted just underneath the naevus, expanding the skin area. Subsequently the naevus was excised and the wound was closed with the overlapping expanded full-thickness skin. (Ronert, 2003).

1.5.2.1 Engineering a dermo-epidermal substitute

A central goal of the Tissue Biology Research Unit is the development of a full thickness skin substitute that allows the reconstitution of all spatial, functional and cosmetic properties of normal skin. In addition, scarring should be minimized.
The aim is the development of dermo-epidermal skin substitutes. These substitutes consist of human dermal fibroblasts seeded in collagen type I hydrogels as the dermal part, and of human keratinocytes seeded on top of the hydrogels as the epidermal compartment (Fig. 19 A).

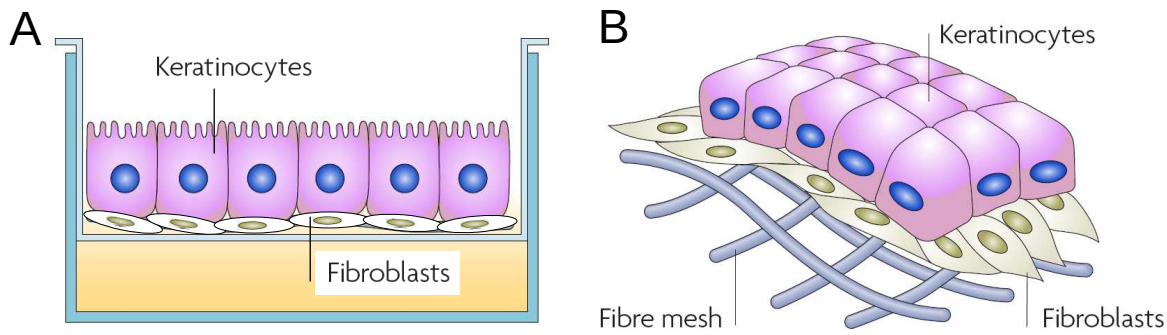

Figure 19: Three-dimensional culture of dermo-epidermal skin substitutes.
A) Schematic representation of a dermo-epidermal substitute grown at air-liquid interface in a cell culture insert. The main disadvantage of this method is the limited diameter in which the substitutes can be grown.
B) For future use in large burns or for reconstructive surgery, larger sizes of substitutes are necessary. A biodegradable scaffold (fibre mesh) can ensure a greater stability, an important concern for clinical application of skin substitutes. (Pampaloni, 2007).

Indeed, we succeeded in developing such dermo-epidermal substitutes. They were grown in cell culture inserts before being transplanted onto the backs of athymic immuno-incompetent rats.

Using this technique, we also aimed at searching for tools suited to evaluate the quality and self-renewing potential of the transplanted engineered human dermo-epidermal skin grafts (Pontiggia, 2009). We found a distinct population of K19 (cytokeratin 19) positive keratinocytes in the basal layer of engineered, stratified skin substitutes. The clustered pattern of these keratinocytes corresponds to the distribution one would expect for self-renewing keratinocytes in the stratum basale.

Concomitantly, there are several reports stating that K19-expressing keratinocytes represent self-renewing keratinocytes (Lane, 1991, Michel, 1996).
Furthermore, transplanted grafts exhibited a continuous layer of basal cells expressing K15 (cytokeratin 15), with K19-positive cells representing a subpopulation of these.
In very young human skin (from neonate to 1.5-year old) K15 was expressed in all keratinocytes of the stratum basale, whereas in the skin of older patients it was expressed in cells of the lower parts of the rete ridges (Pontiggia, 2009). Suprabasal keratinocytes did not show K15 expression. These data are in accord with previous studies (Waseem, 1999, Ghali, 2004, Porter, 2000, Webb, 2004) and imply that in human interfollicular epidermis, K15 is not necessarily a stem cell marker but rather a marker for basal keratinocytes anchored to a functional basement membrane.
In summary it can be said that K19/K15-double-positive keratinocytes represent a distinct basal-cell population in growing skin. For engineered skin substitutes, K19 and K15 in combination are valuable tools to monitor tissue homeostasis and the potential to self-renew (Pontiggia, 2009).

1.5.2.2 Eccrine sweat gland cells can reconstitute a stratified human epidermis

Keratinocyte stem cells are supposed to assure the homeostatic renewal of the epidermis and to be responsible for re-epithelialization of the epidermis after skin injuries. Keratinocyte stem cells reside not only in the epidermis but also in appendageal structures protruding into the dermis. Outer root sheath cells of hair follicles were shown to be able to regenerate a fully differentiated epidermis in vitro and were used for the production of epidermal equivalents for the treatment of skin damage or disease (Lenoir, 1988, Limat, 2003).
We reasoned that human eccrine sweat glands may be an additional source of epidermal stem cells, which may contribute to the in vitro maintenance and long-term survival and function of engineered epidermal grafts. There are some indications that sweat gland cells may have a certain capacity to form an epithelium in culture and in vivo (Lobitz, 1954, Miller, 1998). This fact prompted us to ask whether sweat gland derived epithelial cells can differentiate into keratinocytes and form an epidermis.
To address this issue, we raised organotypic cultures consisting of a dermal component and a stratified epidermal substitute formed by sweat gland cells. These

sweat gland derived substitutes were transplanted onto the backs of immuno-incompetent rats.

The expression of protein markers indicative of the grade of differentiation and homeostasis was determined (Biedermann, 2010). They exhibit almost all the properties of an epidermis derived from a priori epidermal keratinocytes. This is substantiated by the following findings: (1) a stratified epidermis consisting of 10–12 cell layers is formed by sweat gland cells; (2) a distinct, anuclear stratum corneum develops and is maintained after transplantation of sweat gland derived epidermal substitutes in vivo; (3) proteins centrally involved in the process of cornification, such as filaggrin, loricrin, involucrin, envoplakin, periplakin, and the transglutaminases I and III, are expressed in a pattern that matches that of normal human skin; (4) junctional complexes and hemidesmosomes are readily and regularly established; (5) K15 and K19-expressing keratinocytes become expressed in a distinct stratum basale 3 weeks after transplantation (K19-expressing cells disappear 7 weeks after transplantation.); (6) cell proliferation in the basal layer reaches homeostatic levels; (7) the sweat gland derived epidermis is anchored within a well-developed basal lamina; (8) palmo-plantar or mucosal markers are not expressed in the sweat gland-derived epidermis; (9) similar to keratinocyte derived epidermal substitutes, engineered sweat gland cell derived epidermal substitutes undergo maturation after their transplantation onto a living organism. Although with a delay in comparison with epidermal keratinocytes, sweat gland derived cells developed ‘‘de novo’’ into a functional stratified, interfollicular epidermis (Biedermann, 2010).

A crucial point for a future application of our dermo-epidermal skin substitutes is the size of the grafts. We can now increase the dermo-epidermal constructs in size for clinical application (Braziulis, manuscript in preparation). A biodegradable mesh (Fig. 19 B) can be integrated into a collagen I or fibrin hydrogel to stabilize this for easier handling and suturing by the surgeon. We already started to transplant these substitutes using autologous porcine cells onto pigs in a clinical applicabable size (Fig. 20). Hence, after successful termination of these preclinical studies, our novel dermo-epidermal skin substitutes are going to be transplanted onto patients.

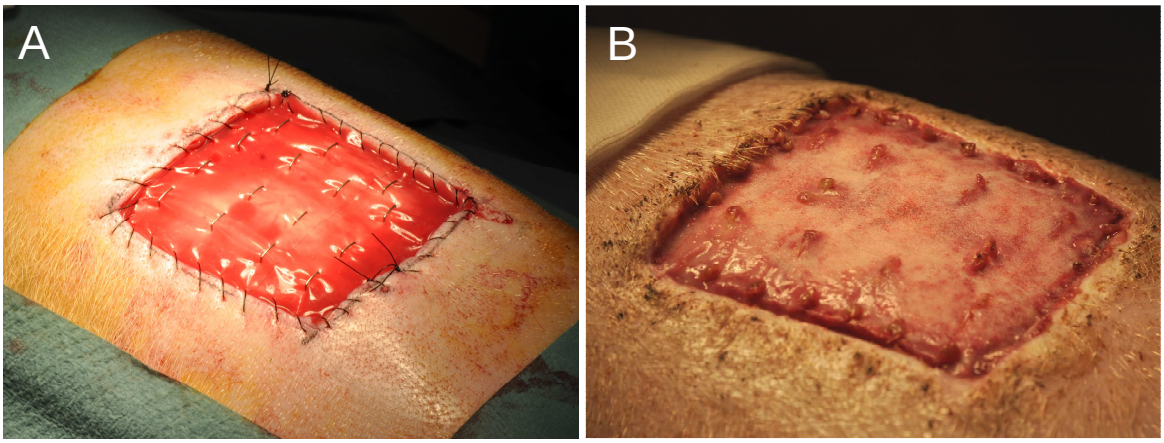

Figure 20: Preclinical study of large scale dermal skin substitutes.
A) As example the application of an artifical dermal substitute (Integra) onto the back of a pig is shown. A 7 x 7 cm full-thickness wound was prepared. Integra was applied and sutured. B) Wound control three weeks after application. Note the excellent quality of the neodermis.

1.5.2.3 The application of skin tissue engineering for melanoma research

Importantly, our dermo-epidermal skin grafts can also be employed to study the biology of skin tumors (Hendrixs 2003, Burdett, 2010). In this respect, primary human melanoma cells were used to produce skin grafts followed by the transplantation onto immuno-incompetent rats (Fig. 21). This *in vivo* tumor model facilitates the study of melanoma cell biology e.g. the process of penetrating the basement membrane and migrating into the dermis (Biedermann, manuscript in preparation). The still unsolved question, if there exist proliferative or migratory melanoma stem cells, can be asked. Possibly, our skin system would also allow preclinical evalution of anticancer drugs. The superiority of this tumor model compared to *in vitro* spheroid cultures or *in vivo* subcutaneous xenografts, is the fact that real melanomas can develop in human skin, and here very specifically in the stratum basale, and that melanoma cell proliferation and invasion can be studied in an authentic environment.

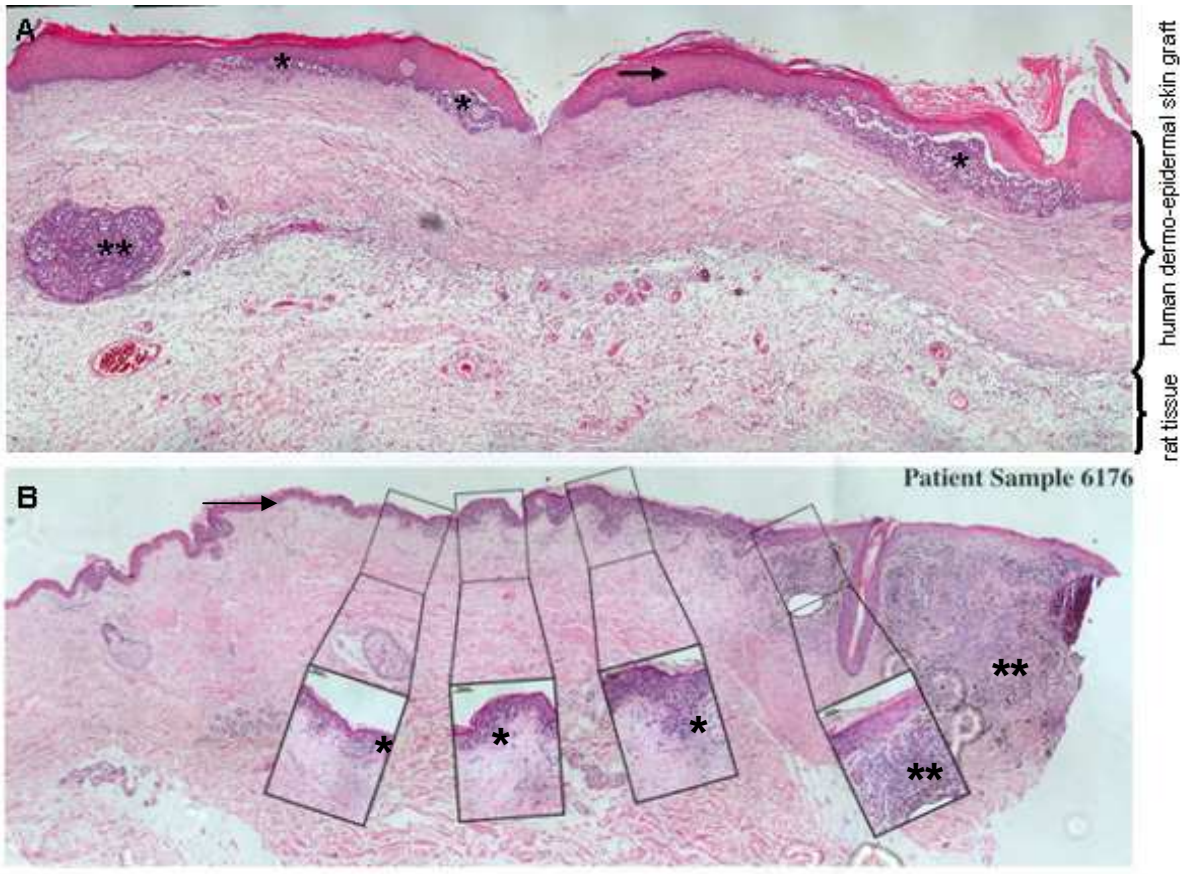

Figure 21: *In vivo* 3-dimensional tumor model.

A) Hematoxylin and eosin staining of a human dermo-epidermal skin graft. Primary human melanoma cells were seeded together with human keratinocytes on top of a collagen hydrogel that was previously populated by human dermal fibroblasts. The engineered tumorigenic skin was transplanted onto immuno-incompetent rats. The black arrow denotes the epidermis. Human melanoma cells are located at the dermo-epidermal junction (black star) and in the dermis (black stars).

B) Hematoxylin and eosin staining of a primary human melanoma as compared to the skin graft melanoma model in A).

2 Results

2.1 Human Eccrine Sweat Gland Cells Can Reconstitute a Stratified Epidermis

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Abstract

Eccrine sweat glands are generally considered to be a possible epidermal stem cell source. Here we compared the multilayered epithelia formed by epidermal keratinocytes and those formed by eccrine sweat gland cells.

We demonstrated both in vitro and in vivo the capability of human eccrine sweat gland cells to form a stratified interfollicular epidermis substitute on collagen hydrogels. This is substantiated by the following findings: (1) a stratified epidermis consisting of 10–12 cell layers is formed by sweat gland cells; (2) a distinct stratum corneum develops and is maintained after transplantation onto immuno-incompetent rats; (3) proteins such as filaggrin, loricrin, involucrin, envoplakin, periplakin, and transglutaminases I and III match with the pattern of the normal human skin; (4) junctional complexes and hemidesmosomes are readily and regularly established; (5) cell proliferation in the basal layer reaches homeostatic levels; (6) the sweat gland-derived epidermis is anchored by hemidesmosomes within a well-developed basal lamina; (7) palmo-plantar or mucosal markers are not expressed in the sweat gland-derived epidermis. These data suggest that human eccrine sweat glands are an additional source of keratinocytes that can generate a stratified epidermis. Our findings raise the question, to what extent the human skin is repaired and/or permanently renewed by eccrine sweat gland cells.

Introduction

Keratinocyte stem cells are supposed to assure the homeostatic renewal of the epidermis and to be responsible for reepithelialization of the epidermis after skin injuries. Keratinocyte stem cells reside not only in the epidermis but also in appendageal structures protruding into the dermis. Outer root sheath cells of hair follicles were shown to be able to regenerate a fully differentiated epidermis in vitro and were used for the production of epidermal equivalents for the treatment of skin damage or disease (Lenoir et al., 1988; Limat et al., 1996, 2003).

The bulge region of both mouse and human hair follicles has been shown to contain a multipotent stem cell population (reviewed in Alonso and Fuchs, 2003; Cotsarelis, 2006). In the mouse, these stem cells do not appear to contribute to epidermal regeneration under homeostatic conditions (Ito et al., 2005; Levy et al., 2005). It remains to be investigated whether this is also true for the human skin. Interestingly, human glabrous skin, which is completely free of hair follicles but contains a high number of sweat glands (Frinkel and Woodley, 2001), also exhibits regenerative potential. This fact prompted us to ask whether sweat gland-derived epithelial cells can differentiate into keratinocytes and form an epidermis.

Human eccrine sweat ducts develop at around gestational week 15–20 by budding from the basal cell layer. The resulting anlagen protrude into the dermis, forming the globular secretory domain (Moll and Moll, 1992; Ersch and Stallmach, 1999). As very little is known about the differentiation of sweat glands, any effort to regenerate them in vitro remains extremely ambitious (Shikiji et al., 2003). There are some indications that sweat gland cells may have a certain capacity to form an epithelium in culture and in vivo (Lobitz et al., 1954; Jones et al., 1988; Miller et al., 1998). Yet, these few studies did not address the issue of the type and quality of the epithelium produced. The presence of a sufficient number of viable epidermal stem cells in cultured epidermal autografts is of major clinical importance (O'Connor, 1981; Auger et al., 2004; De Luca et al., 2006; MacNeil, 2007). We reasoned that human eccrine sweat glands may be an additional source of epidermal stem cells, which may contribute to the in vitro maintenance and long-term survival and function of engineered epidermal grafts.

To address this issue, we raised organotypic cultures consisting of a dermal component and a stratified epidermal substitute (ES) formed by epidermal keratinocytes and sweat gland cells, respectively. These substitutes, designated

KdES (keratinocyte-derived ES) and SdES (sweat gland derived ES), respectively, were transplanted onto the backsof immuno-incompetent rats and the expression of protein markers indicative of the grade of differentiation and homeostasis was determined. Although with a delay in comparison with epidermal keratinocytes, sweat gland-derived cells developed ‘‘de novo’’ into a functional stratified, interfollicular epidermis.

Results

Sweat gland derived epithelial cells acquire properties of epidermal keratinocytes in engineered dermo-epidermal substitutes

Human eccrine sweat glands were isolated from the scalp, abdomen, and the retro-auricular area of young patients ranging in age from 1 to 18 years. As depicted in Figure 1a, preparations of glandular structures consisted of the secretory domain (white arrowheads), the intraglandular duct, and stretches of the intradermal duct of varying length (black arrows). Isolated intradermal ducts always lacked the intraepidermal acrosyringium and a significant stretch of the upper duct (Figure 1a). Consequently, epidermal cells were avoided in sweat gland-derived cell preparations. Keratinocytes were isolated mainly from foreskins. Both cell types were expanded during passage 1. In Figure 1b, the outgrowth of the above-mentioned three domains of an eccrine sweat gland is depicted. Myoepithelial cells were associated with secretory sweat gland cells and were co-isolated. They were detected by smooth muscle actin expression and shown to disappear from the culture within 5–7 days (data not shown).

In the subsequent experiments, sweat gland-derived (three-dimensional) cell cultures were compared with the corresponding cultures of keratinocytes. The cells were initially seeded within a ring of 0.7cm^2, and were grown on collagen type I hydrogels that had been previously populated with 1x10^5 primary human dermal fibroblasts. Keratinocytes finally covered the entire gel, thereby showing a surface expansion of about 4 times (Figure 1c). Sweat gland cells expanded somewhat slower, increasing the surface of the initial cell layer by approximately 2.5 times (Figure 1d).

After the in vitro culture phase, the composite substitutes consisting of a dermal and a multilayered epidermal equivalent were transplanted onto the backs of immunoincompetent Nu/Nu rats. All the subsequent comparative analyses were performed on histological sections of dermo-epidermal grafts after their transplantation onto immuno-incompetent rats. To prevent the ingrowth of rat-derived skin cells, a transplantation chamber was used as previously described (Pontiggia et al., 2009; Figure 1e and f). Histological analyses 21 days after transplantation revealed that both epidermal keratinocytes and sweat gland cells formed a stratified epidermis, which consisted of a basal layer, 10–12 suprabasal layers, and a well-differentiated anuclear stratum corneum (Figure 1g and h).

Characteristics of a stratified epidermis formed by sweat gland cells

Owing to the use of the microdissection technique, it is unlikely that our sweat gland cell preparations were contaminated by epidermal keratinocytes. Nevertheless, we performed control experiments to discriminate between keratinocytes and sweat gland cells in the initial cell preparations (Figure 2c and d) and in SdES and KdES (Figure 3a–f). As shown in Table 1, only a very limited selection of antibodies, such as antibodies to K2e and K8, could be used for these analyses. K2e is expressed in all suprabasal keratinocytes of the human epidermis (Smith et al., 1999), whereas it cannot be recognized in the sweat gland, not even in the intraepidermal acrosyringium (Figure 2a and b). As shown in Figure 2b, K8 expression is confined to the secretory domain of the sweat gland.

Cytospins of newly prepared primary sweat gland cells and keratinocytes were analyzed for the expression of K2e and K8. Importantly, none of the sweat gland cell preparations (n=3) contained K2e-expressing cells, whereas about 50% were positive for K8 (Figure 2d). These findings indicate that the sweat gland cell preparations were not contaminated by (suprabasal) epidermal keratinocytes. In contrast, the majority of epidermal keratinocytes expressed K2e, whereas these cells did not stain for K8 (Figure 2c). Although epidermal keratinocytes completely downregulated K2e expression on cell culture plastic (data not shown), a significant number of them re-expressed K2e when integrated into differentiated ESs, whereas K8 was not expressed by epidermal keratinocytes (Figure 2e).

One significant difference between human eccrine sweat gland cells and epidermal keratinocytes was that only a relatively low number of sweat gland cells expressed K2e within 5–7 weeks in differentiating epidermal equivalents (Figure 2f). Notably, the number of K2e-expressing cells in SdES did not significantly increase 7–10 weeks after their transplantation (data not shown). Thus, the K2e-expressing cells in SdES may represent sweat gland-derived cells that underwent additional epidermal differentiation steps. It remains to be investigated, however, whether the number of K2e-positive sweat gland cells would still increase during an extended in vivo differentiation period of ≥3 months. Notably, K8-positive cells were completely absent in SdES, indicating that this (secretory domain-specific) sweat gland marker was no longer expressed in the engineered epidermis (Figure 2f).

In the next experimental series we set out to explore whether the SdES exhibited mucosal or palmo-plantar characteristics. This was done by screening the two types

of ESs for the expression of K4, a mucosal marker, and K9, a marker of palmo-plantar epidermis. In none of these experiments (n=4) did we find expression of K4, indicating that there was no mucosal differentiation. Furthermore, K9-positive cells were detected neither in KdES nor in SdES (n=4), showing that palmo-plantar properties were absent (Figure 2g and h).

To monitor the contribution of rat keratinocytes to the formation of the ES, we intended to confirm the human origin of the substitutes. Paraffin sections of engineered transplants were subjected to in situ hybridization using DNA probes specifically hybridizing to primate-specific Alu repeats. Figure 2i shows the well-defined transition zone (black dotted line) between the graft of human origin and the underlying, unstained rat tissue. Immunostaining for the human-specific nuclear antigen and for the human fibroblast specific CD90/Thy-1 antigen also confirmed the human origin of the analyzed tissue sections (data not shown).

As there was no reliable antibody allowing to discriminate between basal epidermal keratinocytes and sweat gland cells (see also Table 1), we used an indirect assay to determine whether cells of the epidermal stratum basale were ‘‘contaminating’’ the sweat gland cell fraction. This was achieved by screening the two different types of ESs (KdES and SdES) for the presence of epidermal melanocytes. Melanocytes arelocated in the epidermal stratum basale, and are always present in epidermal keratinocyte preparations, whereas they are absent in eccrine sweat glands. We were able to show that melanocytes were present in all KdESs investigated (Figure 3a), whereas no melanocytes could be detected in SdESs (Figure 3b). Accordingly, we showed that the transfer of melanin from melanocytes to keratinocytes takes place in KdES (Figure 3c and e), whereas this was not possible in SdES (Figure 3d and f).

Homeostasis of transplanted epidermal substitutes.

To determine the proliferative capacity of the epithelial cells in the ESs, frozen sections were stained for Ki-67, a nuclear cell proliferation-associated antigen, which is expressed in all active stages of the cell cycle (Gerdes et al., 1991). In unwounded, homeostatic human skin, Ki-67 was expressed in single keratinocytes in the basal and first suprabasal layers in the interfollicular epidermis (Supplementary Figure S1a). We observed Ki-67-positive cells in the basal layer of both types of ESs (Figure 4a and b) 3 weeks after transplantation, indicating that the stratification process was

ongoing and that, as in normal skin, only a small subpopulation of keratinocytes maintained epidermal renewal. An identical pattern of proliferative cells was observed in 7-week-old grafts (Figure 4c).

Normal, homeostatic interfollicular epidermis lacks the expression of K16 and K17 (Moll et al., 1983; Troyanovsky et al., 1989; see also Supplementary Figure S1a online). These keratins are induced in suprabasal, post-mitotic keratinocytes when tissue homeostasis is perturbed in wound-repair situations, such as the re-epithelialization after transplantation of dermo-epidermal substitutes or in cultures of dissociated keratinocytes grown on cell culture plastic (Leigh et al., 1995; Paladini et al., 1996; Coulombe, 1997). In both KdESs and SdESs, the expression of K16 was clearly detected in suprabasal cell layers soon after transplantation (Figure 4a and b), indicating a persistent wound-healing activity. At about 7–10 weeks after transplantation K16 was somewhat downregulated in both types of substitutes (Figure 4c; data not shown).

Stratification of engineered epidermal equivalents

Involucrin, envoplakin, and periplakin are markers of the cornification process and are associated with the formation of desmosomes and intermediate filaments (DiColandrea et al., 2000). In normal human skin involucrin and loricrin are expressed in the upper granular and cornified layers (Supplementary Figure S1c online).

We found that the ‘‘early’’ pattern of involucrin expression differed from that of homeostatic skin in both types of substitutes. In fact, a strong expression was visible in the spinous layer 3 weeks after transplantation (Figure 4d and e). At 7 weeks after transplantation, as stratification and tissue homeostasis had further progressed, the lower suprabasal layers had downregulated involucrin expression (Figure 4f). The expression pattern of loricrin was similar to that of normal skin, both in the KdESs and in the SdESs, 3 weeks (Figure 4d and e) and 7 weeks (Figure 4f) after transplantation. Expression of envoplakin, filaggrin, and the transglutaminases I and III essentially confirmed the findings obtained with loricrin and involucrin (data not shown). Once again, the differences between KdESs and SdESs were moderate.

The distribution of K1/10, as late differentiation markers (Patel et al., 2006), is indicative of the degree of tissue homeostasis in normal human skin. In fact, during the stratification process K10 appeared sooner in all suprabasal layers, whereas K1

expression was delayed and started in the more upper layers (Stark et al., 1999). KdES (Figure 4g) eventually showed a staining pattern comparable to that of normal skin (see also Supplementary Figure S1d online). In general, the stratification process of the SdES was delayed in comparison with the KdES, with K1 being expressed after 3 weeks only in the upper stratum spinosum and granulosum (Figure 4h). In SdES the expression pattern of normal human skin was reached at about 7 weeks after transplantation (Figure 4i).

During stratification the epidermis establishes an intricate network of different intercellular junctional complexes to establish its functions as a barrier against external agents, as a water-balance regulator, and as a nutrient circulator (Morita and Miyachi, 2003; Perez-Moreno et al., 2003; Dusek et al., 2007). We analyzed the presence of tight junctions, as, together with the lamellar bodies of the cornified envelope, these establish the epidermal diffusion barrier (Malminen et al., 2003; Schluter et al., 2004). One of the major constituents of tight junctions, occludin, is expressed in the upper granular layer of the human skin (Supplementary Figure S1e). Its typical expression pattern was obtained in both types of ES, 3 weeks after transplantation (Figure 4k and l). However, in the SdES we observed stronger and more homogeneous occludin expression only at 7 weeks after transplantation (Figure 4m).

E-cadherin is expressed as a component of adherens junctions in the living keratinocyte layers of the human epidermis (Moles and Watt, 1997; see also Supplementary Figure S1e online). Both types of ESs showed the expression pattern of a normal epidermis (Figure 4k, l, and m).

Desmosomes and the dermo-epidermal junction in engineered SdES

The desmosomal proteins desmoglein (Dsg) 1 and 3 are expressed in a graded manner in the human epidermis. Dsg 3 is expressed in the stratum basale, whereas Dsg 1 can be detected in the upper layers (Supplementary Figure S1f online). Dsg 1 and 3 and their partners desmocollin 1 and 3, respectively, are involved in the regulation of epidermal differentiation (Elias et al., 2001; Dusek et al., 2007). Dsg 3 is expressed exclusively in the ductal portion of the sweat gland (data not shown). During the first 3 weeks after transplantation both types of substitutes showed a somewhat unbalanced (mixed) expression pattern of Dsg 1 and Dsg 3. The suprabasal layers co-expressed both proteins in comparable quantities (Figure 5a

and b). For the establishment of the physiological protein gradients, longer time periods (7 weeks and more) were necessary (Figure 5c).

Periplakin was detected in all suprabasal layers, but was more prominent in the upper spinous and granular layers (Figure 5d and f, and Supplementary Figure S1b online). Once again, this pattern was not yet completely established in SdES after 3 weeks (Figure 5 e).

Integrin α6β4 has a central role in the binding of hemidesmosomes to laminin 332 (laminin 5), which is an important constituent of the basement membrane on which basal epidermal keratinocytes reside (Aumailley and Rousselle, 1999).

Integrin α6 (Figure 5d, e, and f) and laminin 332 (laminin 5, Figure 5g, h, and i), as well as laminin 511 (laminin 10, data not shown), were found to be expressed in the basement membranes of KdES and SdES. For both proteins the expression pattern did not differ from that of normal skin (for comparison, see Supplementary Figure S1b and g online). However, in SdES, homogeneous expression was delayed.

K5 is expressed in the basal layer and the first suprabasal layers in normal human homeostatic epidermis (Supplementary Figure S1g online). However, expression of K5 can be induced in migrating (activated) keratinocytes at wound edges (Patel et al., 2006). Our analyses revealed that in KdES almost the normal staining pattern was found after 3 weeks (Figure 5g), whereas in SdES, K5 was strongly expressed suprabasally (Figure 5h). At 7 weeks after transplantation both types of ESs showed a K5 distribution that was similar to that of normal human epidermis (Figure 5i).

K15 is a useful indicator of epidermal homeostasis, as it is almost exclusively expressed in basal keratinocytes in a homeostatic epidermis (Pontiggia et al., 2009). Indeed, it is absent during wound healing (Waseem et al., 1999; Porter et al., 2000). Consistently, K15 was neither expressed in keratinocytes and sweat gland cells grown on culture plastic, nor in KdES/SdES before transplantation. After transplantation K15 became upregulated in the stratum basale, which then exhibited the K15 expression pattern of normal human skin (Figure 5k, l, and m; compare with Supplementary Figure S1h online).

K19 is considered to be a marker of very ‘‘young’’, proliferative human skin (found in children from less than 2 years) (Pontiggia et al., 2009). In normal interfollicular epidermis, K19-positive keratinocytes are clustered in the stratum basale as a subpopulation of K15-positive basal keratinocytes in young skin and disappear with age (Pontiggia et al., 2009; Supplementary Figure S1h online). Figure 5k and l shows

K19 expression in both KdESs and SdES 3 weeks after transplantation. Note that in Figure 5l some K19-positive keratinocytes are still distributed suprabasally, indicating that tissue homeostasis was not yet completely established. In 7-week-old transplants, K19 expression was already undetectable in both types of substitutes (Figure 5m). The expression patterns of all markers used in this study are summarized in Table 2.

Ultrastructural analysis of the epidermal substitutes

Using transmission electron microscopy, we set out to study the two types of ESs on an ultrastructural level. Figure 6a shows an overview of an SdES, whereas the same structures in KdES are not shown. Quite frequently, blood vessels containing erythrocytes were evident (data not shown). The transplants were generally readily tolerated by the rats. Accordingly, macrophages and leukocytes in the dermal extracellular matrix were rare. Relatively large epidermal intercellular spaces, indicative of active fluid transport and occasional autophagosomes in the epithelial cells (asterisk in Figure 6b), denote a high metabolic turnover in the ESs.

Figure 6b illustrates a sweat gland-derived cell with clearly visible intermediate filament bundles (white arrow). This basal cell formed hemidesmosomes (large black arrows) connecting to the basement membrane (group of small black arrows). The complex organization of hemidesmosomes is illustrated in Figure 6c. In the spinous layer, sweat gland cells interconnected to one another via desmosomes (Figure 6d). All structures indicative of a normal cornified envelope can be detected (Figure 6e): In the upper spinous layer, cells flattened during keratinization and became considerably smaller. Keratohyalin granules (Figure 6e, arrows) were detected in the cells of the granular layer. They increased in number and size toward the stratum granulosum, whereas the nuclei disappeared. Finally, an electron-dense stratum corneum, consisting of flattened cells devoid of organelles, as well as detaching squames were evident. Figure 6f shows the magnification of a lamellar body in contact with the plasma membrane.

Taken together, transmission electron microscopy analyses of SdES revealed that human eccrine sweat gland cells can form a stratified ES, which, ultrastructurally, is indistinguishable from a KdES.

Discussion

A central issue of engineering human skin substitutes is the number and quality of epidermal keratinocyte stem cells present in the keratinocyte preparation. A distinct keratinocyte stem cell pool, referred to as the epidermal proliferative unit (EPU), is commonly thought to be located in the epidermal stratum basale (Ghazizadeh and Taichman, 2005; Kaur, 2006; Strachan and Ghadially, 2008). An additional source of self-renewing keratinocytes is the hair follicle (Alonso and Fuchs, 2003; Cotsarelis, 2006; Blanpain and Fuchs, 2009). However, there is evidence that keratinocyte stem cells are only released from the hair follicle bulge if induced by epidermal wounding (Ito et al., 2005; Levy et al., 2005, 2007). The fact that there are regions of the human body that are devoid of hair follicles, such as palmo-plantar skin, indicate that hair follicles are not an exclusive source for keratinocyte stem cells.

An additional appendage of human skin is the eccrine sweat gland. Almost the entire human body is covered with sweat glands. One may therefore ask whether, in analogy to hair follicles, sweat glands can give rise to interfollicular keratinocytes in wound situations, or even serve as a permanent source of interfollicular keratinocyte stem cells (see also Figure 7).

Using a combined in vitro/in vivo bioassay, we demonstrated that ESs can be generated from sweat gland-derived epithelial cells. These substitutes can be further differentiated and maintained in homeostasis on experimental animals for at least 2 months. They exhibit almost all the properties of an epidermis derived from a priori epidermal keratinocytes (with the exception of K2e expression, which appears to be retarded). This is substantiated by the following findings: (1) a stratified epidermis consisting of 10–12 cell layers is formed by sweat gland cells; (2) a distinct, anuclear stratum corneum develops and is maintained after transplantation of SdES in vivo; (3) proteins centrally involved in the process of cornification, such as filaggrin, loricrin, involucrin, envoplakin, periplakin, and the transglutaminases I and III, are expressed in a pattern that matches that of normal human skin; (4) junctional complexes and hemidesmosomes are readily and regularly established; (5) K15 and K19-expressing keratinocytes become expressed in a distinct stratum basale 3 weeks after transplantation (K19-expressing cells disappear 7 weeks after transplantation.); (6) cell proliferation in the basal layer reaches homeostatic levels; (7) the sweat glandderived epidermis is anchored within a well-developed basal lamina; (8) palmo-plantar or mucosal markers are not expressed in the sweat gland-

derived epidermis; (9) similar to KdESs, engineered SdESs undergo maturation after their transplantation onto a living organism.

It was important to have the sweat gland preparations free of epidermal keratinocytes. This was achieved by using the micro-dissection technique, which was based on optical inspection of every single sweat gland, allowing the careful exclusion of the upper ‘‘epidermal’’ duct in all of the prepared sweat glands. The absence of epidermal tissue was confirmed by showing that K2e-positive keratinocytes, constituting 80–90% of the cells of the human epidermis, were absent in the sweat gland preparations. In addition, we provided indirect indication that no cells of the epidermal stratum basale were ‘‘contaminating’’ the sweat gland cell fraction. As there was no reliable antibody that allowed to discriminate between basal epidermal keratinocytes and sweat gland cells, this was done by demonstrating that epidermal melanocytes were absent in the sweat gland cell fraction. Taken together, these lines of evidence strongly suggest that a pure sweat gland cell population, consisting of secretory and ductal cells, has the potential to develop into a functional, stratified epidermis.

Although SdESs exhibited almost the complete set of epidermal markers and all the features of epidermal differentiation (see also Table 2), we realized a developmental delay in the expression of these markers and the establishment of epidermal homeostasis when compared with KdESs. This is most likely due to sweat gland cells undergoing a timeconsuming transition from ductal or secretory glandular cells to interfollicular epidermal keratinocytes. Thus, the delayed expression of markers such as K1, K10, K5, K19, and occludin in SdES is not really surprising. In addition, myoepithelial cells that were co-isolated with secretory sweat gland cells may have a stabilizing effect on the sweat gland cell phenotype in the organism. In our experimental setting, myoepithelial cells relatively rapidly disappeared in the cell cultures. Thus, the loss of myoepithelial cells, which are closely associated with secretory sweat gland cells, in combination with the massive cell dissociation (representing an extreme wound situation) and the subsequent proliferation on cell culture plastic, may be the prerequisite for the transition of sweat gland cells into epidermal keratinocytes.

Sweat gland cells are derived from ectodermal cells that differentiated into ductal and secretory glandular cells during embryogenesis (Fu et al., 2005). The cell transition

described in this paper may represent the reversion of this ontogenetic process. There are two conceivable mechanisms:

1. A multipotent, sweat gland-derived stem cell population proliferates and the resulting new cells differentiate in response to their new epidermal environment.

2. Sweat gland cells might de-differentiate into a more naive state, after which they differentiate into a new phenotype, i.e., epidermal keratinocytes. Based on the fact that the new sweat gland-derived epidermis reached a state of homeostasis, and was permanently maintained in vivo, it has to be postulated that self-renewing cells assured the integrity of the new epidermis. We consider it therefore likely that both re-programmed sweat gland derived cells and sweat gland-derived stem cells in concert formed and maintained the new epidermis.

Interestingly, under no experimental circumstances did sweat gland-derived epithelial cells develop into sweat glandlike structures. This suggests that the observed cell transition follows a default pathway of differentiation, which invariably causes sweat gland cells to convert into epidermal keratinocytes. This pathway may well be of regulatory relevance. It is consistent with the concept that, in analogy to keratinocytes derived from human hair follicle bulge, a self-renewing subpopulation of sweat gland cells has the potential to reconstitute human epidermis upon wounding, or even to permanently participate in regenerating it (for an illustration, see Figure 7). As glabrous skin (such as the palms and soles) is rich in sweat glands (Frinkel and Woodley, 2001), the presence of a self-renewing, potentially epidermal cell population in sweat glands was repeatedly postulated (Brouard and Barrandon, 2003; Barrandon, 2007). The data presented in this paper provide evidence that significantly supports this assumption. To our knowledge this is previously unreported.

It remains to be investigated which subtype of sweat gland cells, secretory and/or ductal cells, is involved in epidermal regeneration and maintenance. Some recent evidence suggests the presence of a label-retaining, multi-potent stem cell population in the secretory domain of the human eccrine sweat gland (Nakamura and Tokura, 2009). Apart from that, only few data can be found in the literature that support the ability of the human sweat gland cells to form an epithelium in culture and in vivo. Miller et al. (1998) reported that in pigs the ‘‘sweat apparatus’’ can re-epithelialize the skin ‘‘de novo’’ after wounding. These authors concluded that porcine sweat gland cells formed an epithelium that had the morphological and structural features of

palmo-plantar or buccal epithelia, rather than the features of trunk epidermis. However, in contrast to human skin, which contains eccrine sweat glands, porcine skin harbors apocrine glands, the function of which is different from that of human sweat glands (Montagna and Yun, 1964; Ferry et al., 1995).

In conclusion, this is the first study generating comprehensive evidence that a near-normal epidermis can be cultured from sweat gland cells in vitro. Furthermore, the SdES can be successfully transplanted onto immuno-incompetent rats on which they adhere, further differentiate, and survive as a mature graft, which is strikingly similar to the KdES. This study enlarges the understanding of skin biology in that it corroborates the thesis that sweat gland cells can switch their phenotype and become keratinocytes. From a tissue engineering point of view, sweat gland-derived epithelial cells obviously represent an additional source of keratinocytes to grow a near-normal autologous epidermis. This is particularly relevant for patients requiring large and urgent covering of skin defects, such as severe burn injuries (third degree; >50% BSA). In these cases, self-renewing keratinocytes are urgently required for the in vitro production of as many life-saving skin grafts as possible.

Materials and Methods

Preparation of skin cells

This study was conducted according to Declaration of Helsinki Principles. Human skin samples from scalp, abdomen, retroauricular skin or foreskins were obtained from patients ranging in age between 1 and 18 years, after permission by the ethic commission of the Canton Zurich and after informed consent given by parents or patients. The skin samples were used for the isolation of sweat gland cells, keratinocytes, and fibroblasts, or employed for histological analysis.

Establishment of primary cell cultures

Keratinocytes and fibroblasts were isolated as described (Pontiggia et al., 2009). For sweat gland cells, we used the following procedure:

The day before isolation mitomycin-treated or irradiated swiss albino 3T3 mouse fibroblasts (ATCC CCL-92) were seeded at a density of 10'000 per cm^2 in DMEM, 10% calf serum and 5 µg/ml gentamycin. Just before plating the fragments of sweat glands, the medium was changed to Rheinwald and Green medium (RGM) (Jones et al., 1988; Schon et al., 1999), i.e. 3 parts of DMEM and one part of Ham's F12, 10% FCS, 5 µg/ml gentamycin, 1.4 mM $CaCl_2$ (all Sigma, Buchs, Switzerland), 0.4 µg/ml hydrocortisone, 5 µg/ml insulin, 2 nM triiodothyronine, 180 µM adenine, 10 ng/ml EGF (all from Invitrogen, Basel, Switzerland) and 0.1 nM choleratoxin (Calbiochem/VWR International AG, Dietikon, Switzerland). Skin samples were cut into small pieces (~6 mm^2), digested in DMEM containing 12 U/ml dispase (Invitrogen, Basel, Switzerland), 2 mg/ml collagenase blend F (Sigma, Buchs, Switzerland) and 38 U/ml collagenase II (Worthington, Bioconcept, Allschwil, Switzerland) for 16 h at 4℃ and 4 h at 37℃ until the mesenchyme was almost completely digested. The tissue pieces were centrifuged and fat droplets were removed with the supernatant. The pellet was resuspended in 20 ml of DMEM containing 3-fold concentrated antibiotics (gentamycin 15 µg/ml, penicillin 3000 U/ml, streptomycin 3 mg/ml and fungizone 750 ng/ml), 1% FCS and transferred to a new culture dish. The dermal tissue was removed from the epidermis and fragments of sweat glands were collected employing a stereomicroscope and micropipettes. The fragments were washed with culture medium and finally transferred to the culture

dish. At days 4-5 the first outgrowing cells were visible. After two weeks the feeder cells were removed by digestion with 0.1% Trypsin, 1 mM EDTA (Invitrogen, Basel, Switzerland) for 2 min at 37℃. The remaining sweat gland cells were washed twice in PBS and detached from the dish with 0.5% trypsin, 5 mM EDTA, for 5 min at 37℃. Trypsin activity was stopped by the addition of 3.75 mg/ml soy bean trypsin inhibitor (Invitrogen, Basel, Switzerland). An almost single cell suspension was reached by repeated pipetting through a fine pipette tip.

Keratinocytes and sweat gland cells were further expanded during passage 1. Keratinocytes were grown in a serum-free medium (SFM, Invitrogen, containing 0.2 ng/ml EGF and 25 µg/ml Bovine Pituitary Extract). sweat gland cells were maintained in RGM.

Organotypic cultures

Organotypic cultures were prepared using a previously established transwell system consisting of 6 well cell culture inserts with membranes of 3.0 µm pore-size (BD Falcon, Basel, Switzerland) (Pontiggia et al., 2009). The membranes were covered with collagen type I hydrogels containing human dermal fibroblasts (passage 1).

The collagen matrix was prepared according to the protocol of Costea et al. (Costea et al., 2003). Briefly, 0.7 ml of rat tail collagen type I (3.2-3.4 mg/ml, BD Biosciences, Allschwil Switzerland), were added to 0.2 ml chilled neutralization buffer containing 0.15 M NaOH and $1x10^5$ fibroblasts. After polymerization (10 min at room temperature and 20 min at 37℃) these dermal equiva lents were grown in DMEM/10%FCS for 5 days. Subsequently, keratinocytes and sweat gland cells were seeded onto each dermal equivalent at a density of $125x10^3$ cells per cm^2 within siliconized polypropylene rings of 5 mm in diameter to avoid dispersion. After 7h the rings were removed, 1 ml RGM was added in the upper chamber, and 2 ml were added to the lower chamber. Triplicate wells were set up for each dermo-epidermal substitute. The constructs were cultured in RGM. After 4 days the dermo-epidermal substitutes were raised to the air/liquid interface and cultured for 3 additional weeks. The medium was changed every 2nd day. Cultures were finally processed for transplantation or for cryo- and paraffin sections.

Transplantation of cultured dermo-epidermal composites

Dermo-epidermal grafts were transplanted onto full thickness skin defects created surgically and encased by polypropylene rings, 27 mm in diameter as previously described (Pontiggia et al., 2009). The rings were sutured on the back of 10 weeks old, female athymic Nu/Nu rats. The transplants were covered with a silicon foil. After 14 days the grafts were excised and processed for cryo- and paraffin sections. Anesthesia for all procedures was performed using isoflurane (Abbott AG, Baar, Switzerland).

Histology and Immuno-fluorescence

The epidermal substitutes were embedded in OCT compound (Sakura Finetek/Digitana AG, Horgen, Switzerland) and frozen at -20℃.
Melanin was visualized using the Masson-Fontana technique with nuclear fast red as counterstain (Stevens and Chalk, 1996).
Cryosections were fixed and permeabilized in acetone for 5 min at -20℃, air dried, washed 3x in PBS and blocked in PBS, containing 2% BSA (Sigma, Buchs, Switzerland) for 30 minutes. Incubation with the diluted antibodies was performed in blocking buffer for 1 hour at room temperature. Slides were washed three times for 5 min in PBS and blocked for additional 15 min before the second antibody was added. If necessary the same procedure was repeated for the third antibody incubation. Finally the slides were incubated for 5 min in PBS containing 1 µg/ml Hoechst 33342 (Sigma, Buchs, Switzerland), washed twice for 5 min in PBS and mounted with Dako mounting solution (Dako, Baar, Switzerland) containing 25 mg/ml of DABCO anti-quenching agent (Sigma, Buchs, Switzerland).
To stain freshly isolated sweat glands cells the glands were dissociated for 3 min by means of concentrated (3x) trypsin/EDTA (Invitrogen, Basel, Switzerland). After inactivation of trypsin, the cells were fixed in 2% PFA for 10 min, permeabilized with 0.5% saponin for 5 min and incubated with the primary antibodies for 15 minutes on ice. The cells were passed through a 0.2 ml-serum layer by centrifugation and resuspended in PBS containing 2% BSA and the fluorescent-dye- conjugated secondary antibody for 15 min on ice. After a second serum-washing step the cells were centrifuged onto glass slides and the cytospins were analyzed by fluorescence microscopy.

Antibodies

For IF the following antibodies were used: From Dako (Baar, Switzerland): K10 (clone DE-K10, 1:100), E-Cadherin (clone NCH-38, 1:30), laminin 10 (clone 4C7, 1:25), K19 (clone RCK108, 1:100), Melanosome (clone HMB45, 1:50); from Progen (Heidelberg, Germany): K2e (clone Ks 2.342.7.4, 1:100), K9 (polyclonal, 1:200), K5 (polyclonal, 1:100); from Chemicon (Millipore AG, Zug, Switzerland): K16 (clone LL025, 1:100), K1 (clone LHK1, 1:200), α6 integrin (clone 4F10, 1:100), K15 (clone LHK15, 1:100); from Zymed (Invitrogen, Basel, Switzerland): occludin (polyclonal, 1:50), desmoglein 1 (clone 27B2, 1:50), desmoglein 3 (clone 5G11, 1:200); from LabVision (P.H.Stehelin&CIE AG, Basel, Switzerland): involucrin (clone SY5, 1:100), filaggrin (FLG01, 1:100); from Genetex (Biozol, Eching, Germany): K8 (clone B391, 1:200); from Sigma (Buchs, Switzerland): K4 (clone 6B10, 1:600); from BD Pharmingen (Basel, Switzerland): Ki-67 (clone B56, 1:200); from Abcam (Cambridge, UK): loricrin (polyclonal, 1:500); from Santa Cruz (Labforce AG, Nunningen, Switzerland): laminin 5 (clone P3H9-2, 1:100). The periplakin and envoplakin antibodies were a kind gift of Dr. Fiona Watt (Cambridge Research Institute, UK). As a secondary antibody we used FITC-conjugated polyclonal goat $F(ab')_2$ fragments directed to mouse immunoglobulins (Dako, Baar, Switzerland). For double IF, some of the primary antibodies were pre-labeled with Alexa 555-conjugated polyclonal goat F(ab')2 fragments, according to the instructions of the manufacturer (Zenon Mouse IgG Labeling Kit, Molecular Probes, Invitrogen, Basel, Switzerland).

Fluorescence microscopy

Fluorescence microscopy was performed using a Nikon Eclipse TE2000-U inverted microscope equipped with Hoechst, FITC, and TRITC filter sets. For confocal imaging we used a Leica SP1 confocal laser scanning microscope (Leica, Heerbrugg, Switzerland) equipped with Argon UV laser with 351 nm / 364 nm excitation, Argon/Krypton laser with 474 nm / 488 nm / 568 nm / 647 nm excitation, fluorescence filters for DAPI, FITC and TRITC. The line average was set to 4. Images were processed with Imaris 5.0.1 (Bitplane AG, Zurich, Switzerland).

Electron Microscopy

For transmission electron microscopy (TEM) analysis, tissue blocs (approximately 1 mm^3) were prefixed in 0.1 M cacodylate buffer (Merck, Hohenbrunn, Germany), pH 7.3 containing 2.5% glutaraldehyde for 2 hours, washed in cacodylate buffer, postfixed with an aqueous solution of 1% OsO4 and 1.5% K4Fe(CN)6 for 1 hours, dehydrated and finally embedded in EPON 812 (Catalys AG, Wallisellen, Switzerland). Ultrathin sections (approximately 50-70 nm) were collected on copper grids were contrasted with 4% uranyl acetate and 3% lead citrate, and examined with a CM 100 transmission electron microscope (Philips, Eindhoven, The Netherlands). All reagents were from Sigma (Buchs, Switzerland) unless mentioned otherwise.

In situ Hybridisation with Alu-probes

The epidermal substitutes (ES) were fixed in 4% neutral formalin and embedded in paraffin according to standard protocols. We used basically the method published by Just et al. (Just et al., 2003). Briefly: The genomic DNA (gDNA) was extracted with the QIAamp DNA Blood Mini Kit (Qiagen) from the whole blood of a volunteer which donated 2 ml blood after written informed consent. 50 ng of gDNA and 0.5 µM of the primers (Alu-sense: 5' ACG CCT GTA ATC CCA GCA CTT 3'; Alu-antisense: 5' TCG CCC AGG CTG GAG TGC A 3' produced by Microsynth, Balgach, Switzerland) were applied in a PCR with Hot Star Taq DNA polymerase (Qiagen, Hombrechtikon, Switzerland) under the following conditions: 95℃ for 15 min, 25x (94℃ for 30 s, 58℃ for 45 s, 72℃ for 45 s) and 72℃ for 10 min. The 245 bp-product was purified by agarose gel electrophoresis and the QIAquick Gel Extraction Kit (Qiagen). 200 pg were used as template for the DIG-labeling PCR (PCR DIG probe synthesis kit, Roche, Rotkreuz, Switzerland) under the same conditions but with increased $MgCl_2$ concentration (5 mM). The DIG-labelled probe was purified by ethanol precipitation.
Paraffin sections were mounted on superfrost slides, deparaffinized (xylol, 3x 10 min), hydrated (ethanol 100-96-80-70-50%, 2 min each) and washed in PBS (3x 5 min). Cryosections (16 µm thick) were mounted on superfrost slides, dried for 30 min at 37℃, washed in PBS (3x 5 min), fixed with 4% PFA for 20 min and washed again. Both section types were further permeabilized (0.3% Triton X-100 in PBS, 10 min), digested with proteinase K (2 µg/ml in 100 mM Tris/HCl pH 8, 50 mM EDTA, 15 min at 37℃, cryosections 5 min only), acetylated (0.1 M triethanolamine, 0.25% Acetic anhydride, 2x 5 min) and washed first in PBS (2x 5min) and finally in 2xSSC (0.3 M

NaCl, 0.03 M sodium citrate, pH 7.0, 5 min). The hybridisation was performed in a moist chamber with 50% formamide. The slides were incubated in hybridization buffer (50% formamide in 5x SSC, 0.15% N-lauroylsarcosine, 0.02% SDS, 2% blocking reagent) without probe for 30 min at 85℃ and with 100 ng/ml DIG-labelled DNA-Probe for 30 min at 85℃. After chilling on ice for 10 min the slides were incubated over night at 42℃ and finally washed in 2x SSC (briefly) and 0.1x SSC (3x 15 min at 42℃). For staining the sections were blo cked (30 min in 0.15 M NaCl, 0.1 M maleic acid pH 7.5, 1% blocking reagent), incubated with an alkaline phosphatase-conjugated anti-DIG antibody (1:2000 in blocking buffer and 0.1% Triton X-100, 60 min), washed (4x 15 min in 0.15 M NaCl, 0.1 M maleic acid pH 7.5), equilibrated in TBS (0.1 M Tris pH 9.5, 0.1 M NaCl, 5 min), incubated with the substrate solution (200 µl of Nitroblue tetrazolium salt + bromo-chloro-indolyl phosphate in 10 ml TBS, 120 min) and the stop solution (10 mM Tris pH 8.0, 1 mM EDTA, 2 min) and washed in PBS (3x 15 min, second wash containing 1 µg/ml Hoechst 33342). Finally the slides were mounted in Eukitt® quick-hardening mounting medium. All reagents were form Fluka (Buchs, Switzerland); the proteinase K and the DIG nucleic acid detection kit including blocking reagent and substrate solution were from Roche (Rotkreuz, Switzerland).

Conflict of interest

The authors state no conflict of interest.

Acknowledgments

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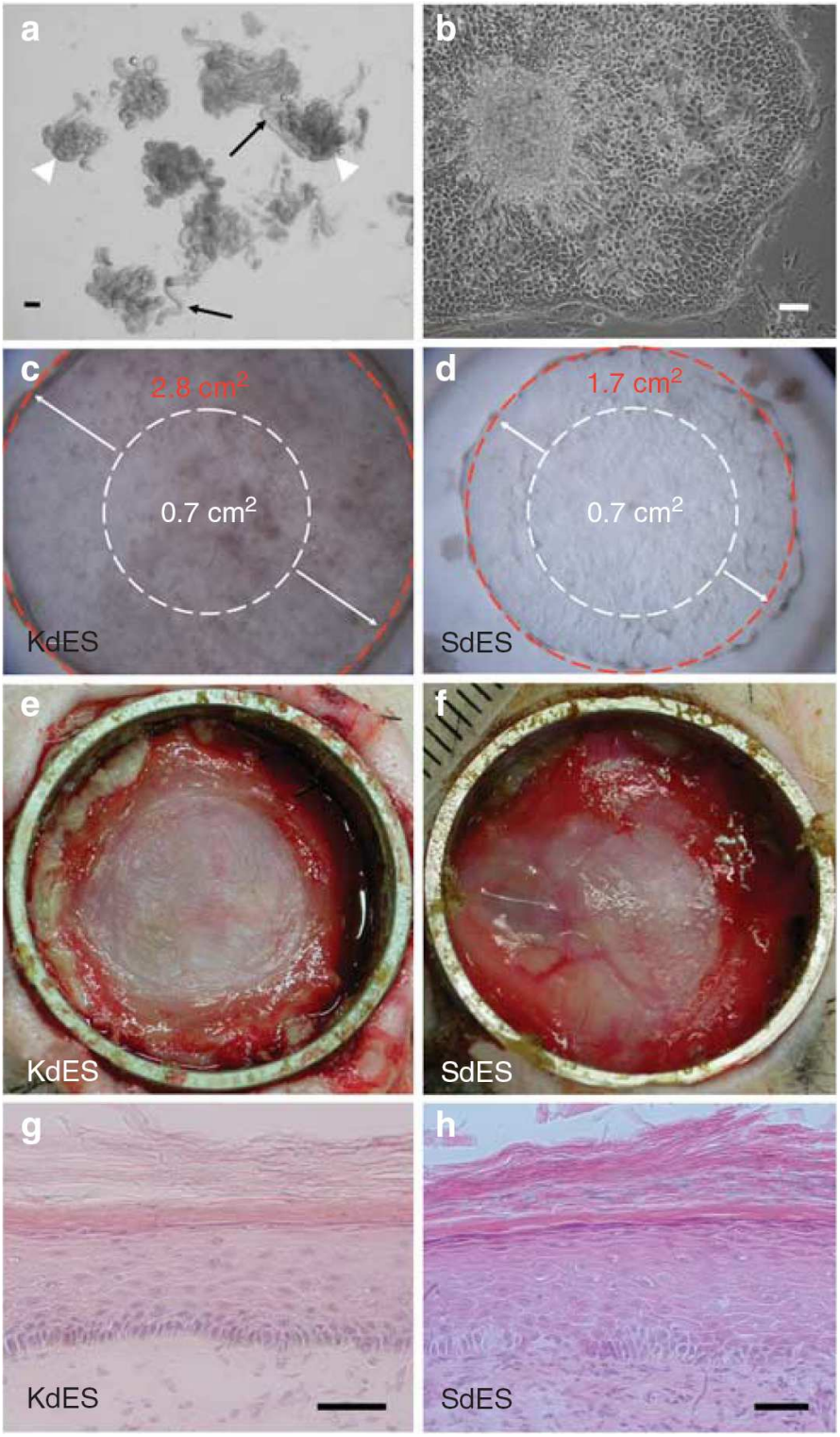

Figure 1. Macroscopic and histologic appearance of SdES and KdES *in vitro* and after transplantation onto immuno-incompetent rats.

(**a**) Isolation of human sweat glands after dispase/collagenase digestion. Sweat gland fragments containing the glandular domain (white arrowheads) and parts of the intradermal ducts (black arrows). (**b**) A typical epithelial cell colony growing out from

a human sweat gland fragment. (**c**, **d**) Keratinocytes and sweat gland cells grown at the air/liquid phase on collagen type I hydrogels on permeable inserts. Keratinocytes were initially seeded within a ring area of 0.7 cm^2. Three weeks after plating this area was increased by about 4 times. Sweat gland cells increased this area by approx. 2.5 times. (**e**, **f**) KdES in e, and SdES in f, three weeks after transplantation onto full thickness skin wounds on the back of athymic rats. (**g**,**h**) Hematoxylin/eosin staining of KdES (g) and SdES (h) three weeks after transplantation reveals a stratified multilayer consisting of a basal layer, 10-12 suprabasal layers and a stratum corneum in both types of substitutes. Scale bars in a) 500 µm, in b) 200 µm, in g, h) 100 µm. The rings in e) and f) are 2.7 cm in diameter.

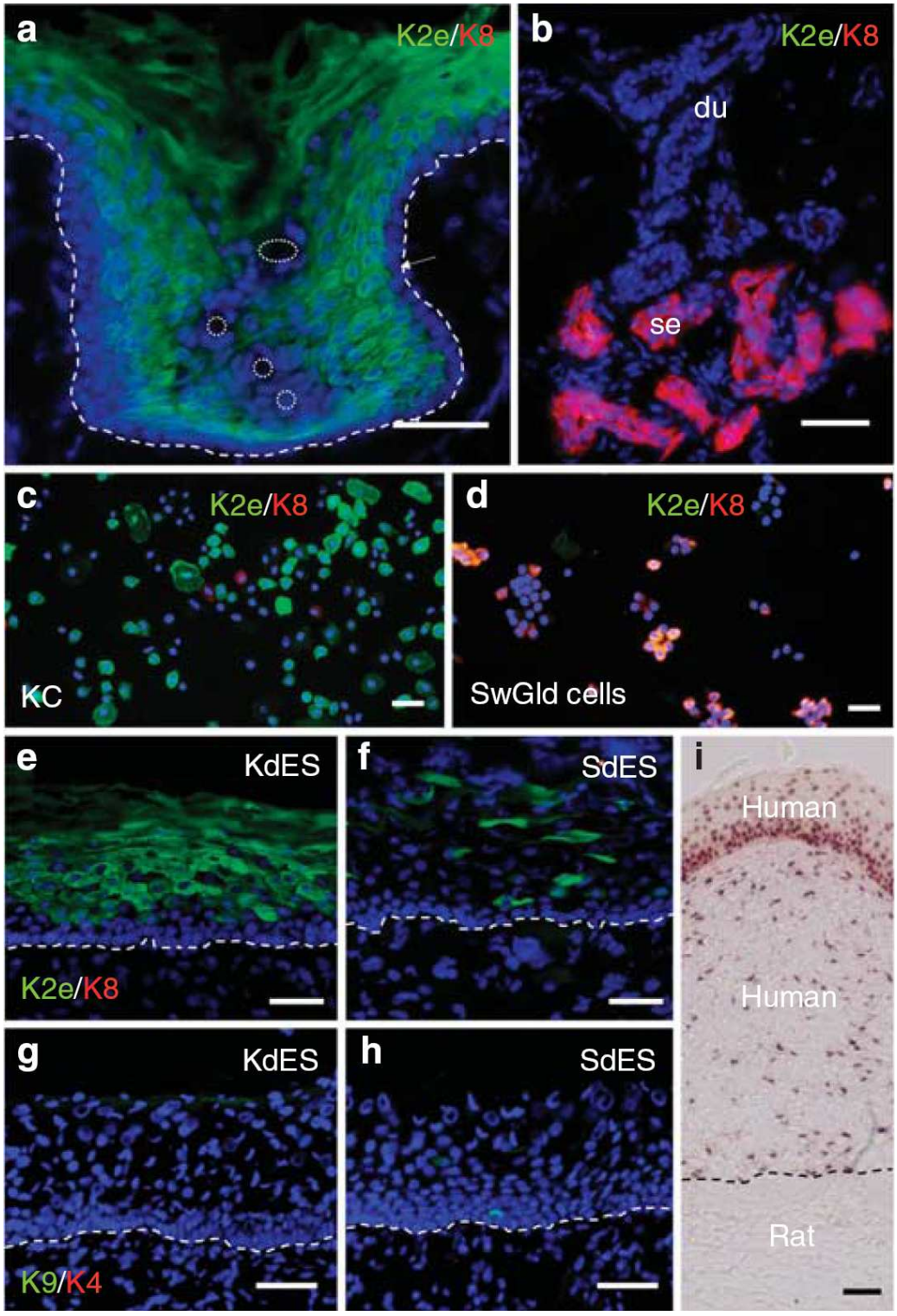

Figure 2. Tissue specific markers in KdES and SdES.

Double immuno-fluorescence using the antibody combinations K2e/K8 (a, b, c, d, e, f) and K9/K4: (g, h). (**a**) K2e is expressed in suprabasal layers (white arrowhead), as demonstrated in the area of the glandular acrosyringium. Note that the sweat gland duct (dotted circles), meandering through the acrosyringium, does not express K2e. K8 is not expressed in normal human epidermis. (**b**) In normal human skin the duct

(du) of an eccrine sweat gland is negative for K8 (and K2e), whereas the secretory domain (se) expresses K8. (**c**) Cytospin of a freshly isolated human sweat gland cell suspension. About 50% of the cells are positive for K8. All sweat gland cells are negative for K2e. (**d**) In contrast, freshly isolated human keratinocytes are positive for K2e and negative for K8. (**e**) Immuno-fluorescence staining for the expression of K2e and K8 of KdES three weeks after transplantation. (**f**) Only a few single cells are staining for K2e/K8 in SdES. (**g**, **h**) Almost no expression of the palmoplantar marker K9 and the mucosal marker K4 in KdES (g) and SdES (h) after transplantation. (**i**) In-situ hybridization of SdES using a DNA-probe that hybridizes to human-specific Alu-sequences. Positive (violet) staining proves the human origin of the dermo-epidermal graft. The boundary between human tissue and the underlying unstained rat tissue is obvious. White dotted line: Dermo-epidermal border. Scale bars for all panels: 50 µm.

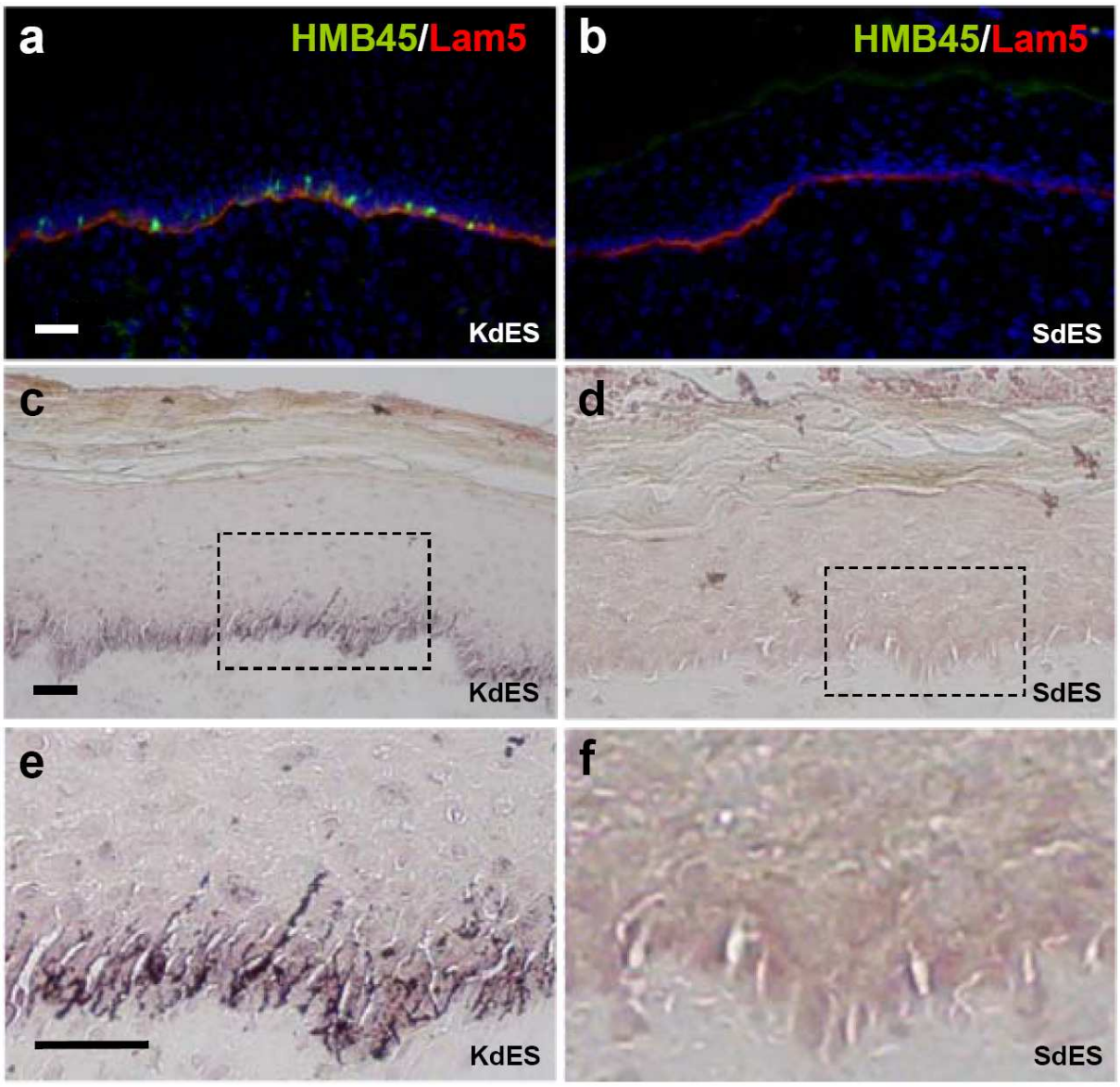

Figure 3. Preparations of human eccrine sweat gland epithelial cells are not contaminated by basal epidermal cells.

a) Histological section of a typical dermo-epidermal substitute (KdES) transplanted onto immuno-incompetent nu/nu rats 3 weeks after transplantation. Melanocytes (HMB45, green cells) located in the stratum basale are obvious. The basement membrane is stained by a laminin 332 antibody. b) No melanocytes are detectable in sections of SdES, indicating that no basal epidermal cells, hence no basal epidermal keratinocytes, were present in the initial sweat gland cell preparation. c) Fontana-Masson staining reveals the melanin content both in basal melanocytes and epidermal keratinocytes. d) No melanosomes are detectable in SdES. e) Magnified detail of c). f) Magnified detail of d). (a-f) Bar = 50 µm.

Table 1. The expression patterns of epithelial markers

Table 1. The expression patterns of epithelial markers

		Human epidermis		Human sweat gland		
Marker	Antibody	Suprabasal KC	Basal KC	Acro-syringium	Duct	Secretory domain
CEA	Polyclonal	–	–	–	+	+
K1	LHK1	+	–	+	–	–
K5	Polyclonal	(+)	+	–	+	+
K8	B391	–	–	–	–	+
K10	DE-K10	+	–	+	+	–
K14	LL002	(+)	+	–	+	+
K16	LL025	+[1]	+[1]	n.d.	+	(+)
K19	RCK108	–	+[2]	+	+	+
Involucrin	SY5	+	–	+	+	–
K1b (K77)	Polyclonal	+	–	–	+	–
K2e	Ks 2.342.7.4	+	–	–	–	–
K15	LHK15, SPM190	–	+	–	–	+
K17	Ks 17.E3	–	–	–	+	+
K20	Ks 20.8	–	–	–	–	–
Desmoglein 1	27B2	+	–	n.d.	–	–
Desmoglein 3	5G11	+	+	n.d.	+	–
Connexin 26	CX-1E8	–	–	–	+	(+)
Integrin α6	4F10	–	+	–	+	+
Aquaporin 5	Polyclonal	–	+	n.d.	–	+
Follistatin	85918	–	+	–	–	+
CD200	MRC OX104	–	–	–	+	–
Na,K-ATPase α1	Polyclonal	+	+	n.d.	+	+
Transglut. 1	Polyclonal	+	–	n.d.	–	–
Transglut. 3	Polyclonal	+	–	n.d.	–	–
Transglut. 5	Polyclonal	+	+	n.d.	+	+

Abbreviations: n.d., not determined; +, expressed; –, not expressed; (+), minimal expression.
[1]Wound healing marker.
[2]New-born.

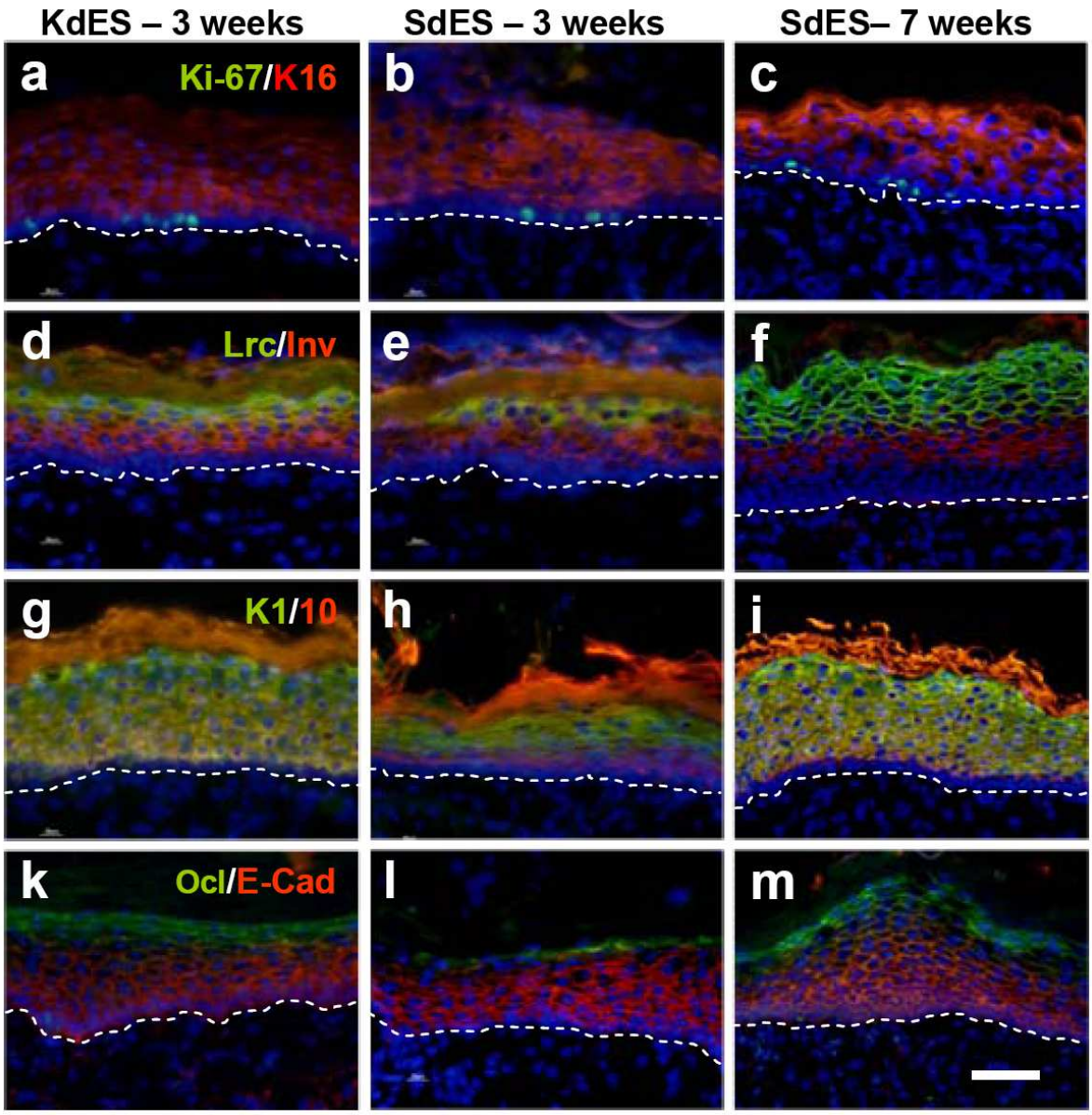

Figure 4. Epidermal stratification and homeostasis in KdES and SdES after transplantation onto immuno-incompetent rats.

Double immuno-fluorescence using antibodies to Ki-67/Cytokeratin 16 (a,b,c), Loricrin/Involucrin (d,e,f), Cytokeratin 1/Cytokeratin 10 (g,h,i), Occludin/E-cadherin (k,l,m). (**a, d, g, k**) Indirect immuno-fluorescence reveals the usual epidermal differentiation of KdES three weeks after transplantation. (**b, e, h, l**) Epidermal differentiation of SdES three weeks after transplantation. A delayed differentiation is obvious with respect to the expression pattern of K1/K10 (h). (**c, f, i, m**) Epidermal differentiation of SdES seven weeks after transplantation. No difference to the control situation (compare to a, d, g, k) can be observed. White dotted line: Dermo-epidermal border. Scale bar for all panels: 50 µm.

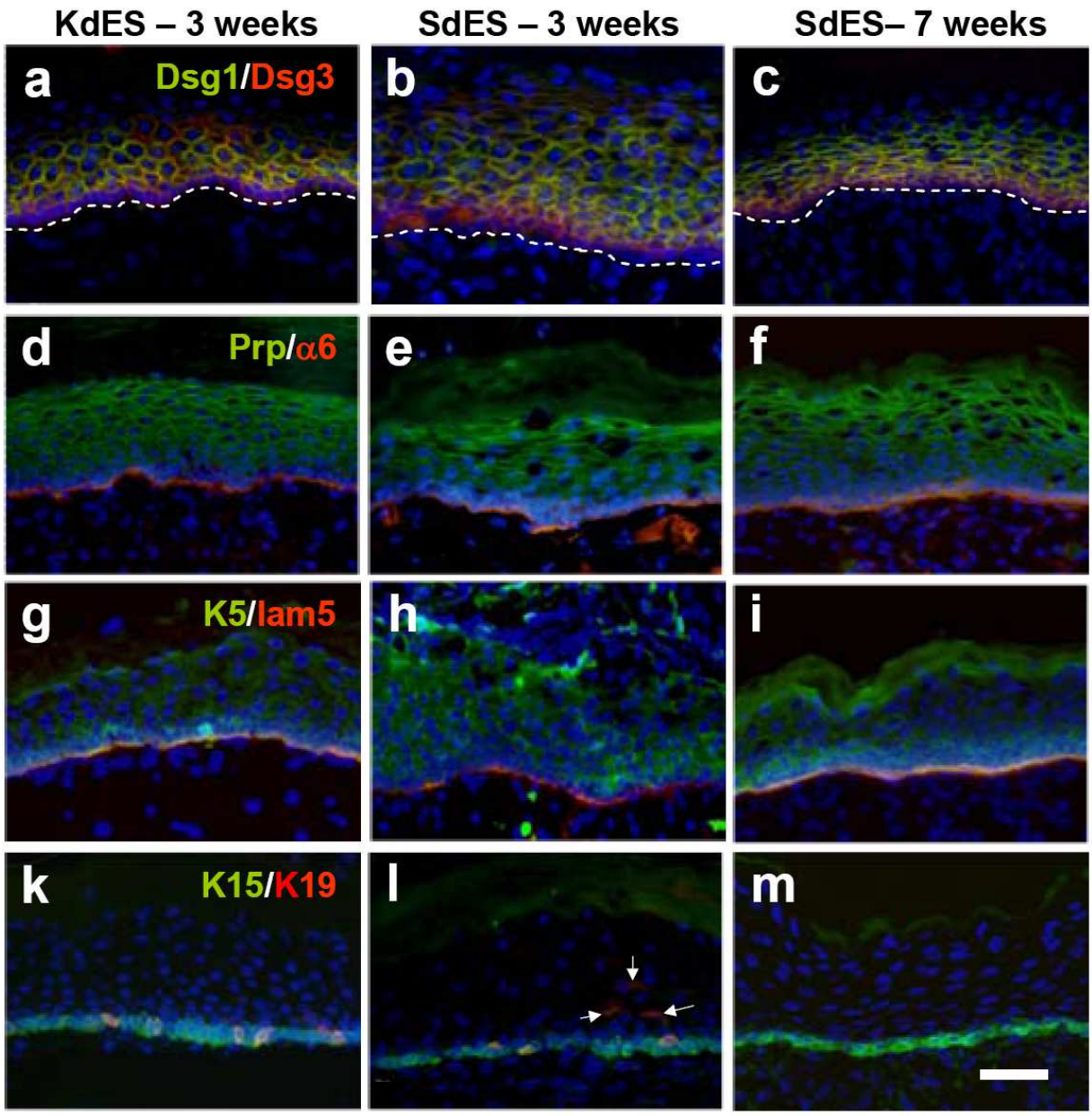

Figure 5. Epidermal stratification and homeostasis in KdES and SdES after transplantation onto immuno-incompetent rats.

Double immuno-fluorescence of cryosections using antibodies to: Desmoglein1/Desmoglein3 (a,b,c), Periplakin/Integrin-α6 (d,e,f), Cytokeratin 5/Laminin 5 (Laminin-332) (g,h,i), Cytokeratin 15/Cytokeratin 19 (k,l,m). (**a, d, g, k**) Indirect immuno-fluorescence displays the typical epidermal differentiation of KdES three weeks after transplantation. (**b, e, h, l**) Epidermal differentiation of SdES three weeks after transplantation. A delayed differentiation is visible with respect to the expression pattern of Dsg1/3 (b), Integrin α6 (e) and K5 (g). Note that some K19-positive keratinocytes are still distributed suprabasally (white arrows in l). (**c, f, i, m**) Epidermal differentiation of SdES seven weeks after transplantation. No difference to

the control situation (compare to a, d, g, k) can be observed. K19-positive keratinocytes in the stratum basale have already disappeared indicating advanced tissue homeostasis and differentiation. White dotted line: Dermo-epidermal border. Scale bar for all panels: 50 µm.

Table 2. Expression of epidermal markers in KdES and SdES

Table 2. Expression of epidermal markers in KdES and SdES

Antigen	Antibody (Clone)	Normal skin	KdES 3 weeks	SdES 3 weeks	SdES 7 weeks	
Occludin	polyclonal	●●	●●	●	●●	
Loricrin	polyclonal	●●	●	●	●	
Involucrin	SY5	●●	●	●	●	
Periplakin	polyclonal	●●	●●	●●	●●	
K1	LHK1	●●	●●	●	●●	
K10	DE-K10	●●	●●	●●	●●	
K2e	Ks 2.342.7.4	●●	●●	(●)	(●)	
K16	LL025	—	●●	●●	●●	
K9	polyclonal	—	—	—	—	Only in palmoplantar skin
Desmoglein 1	27B2	●●	●	●	●	
Desmoglein 3	5G11	●●	●	●	●	
E-Cadherin	NCH-38	●●	●●	●●	●●	
K5	polyclonal	●●	●	●	●	
K15	LHK15, SPM190	●●	●●	●●	●●	
K19	RCK108	●●	●●	●●	—	
Integrin a6	4F10	●●	●●	●●	●●	
Laminin 5	P3H9-2	●●	●●	●●	●●	
Laminin 10	4C7	●●	●●	●●	●●	
Ki-67	B56	●●	●	●	●	
K8	B391	—	—	—	—	Only in secretory sweat gand cells
K4	6B10	—	—	—	—	Only in mucosal epithelium

Abbreviations: ●●, normal expressionl; ●, reduced expression; (●), expressed in single scattered cells; —, no expression; KdES, keratinocyte derived epidermal substitute; SdES, sweat gland derived epidermal substitute.

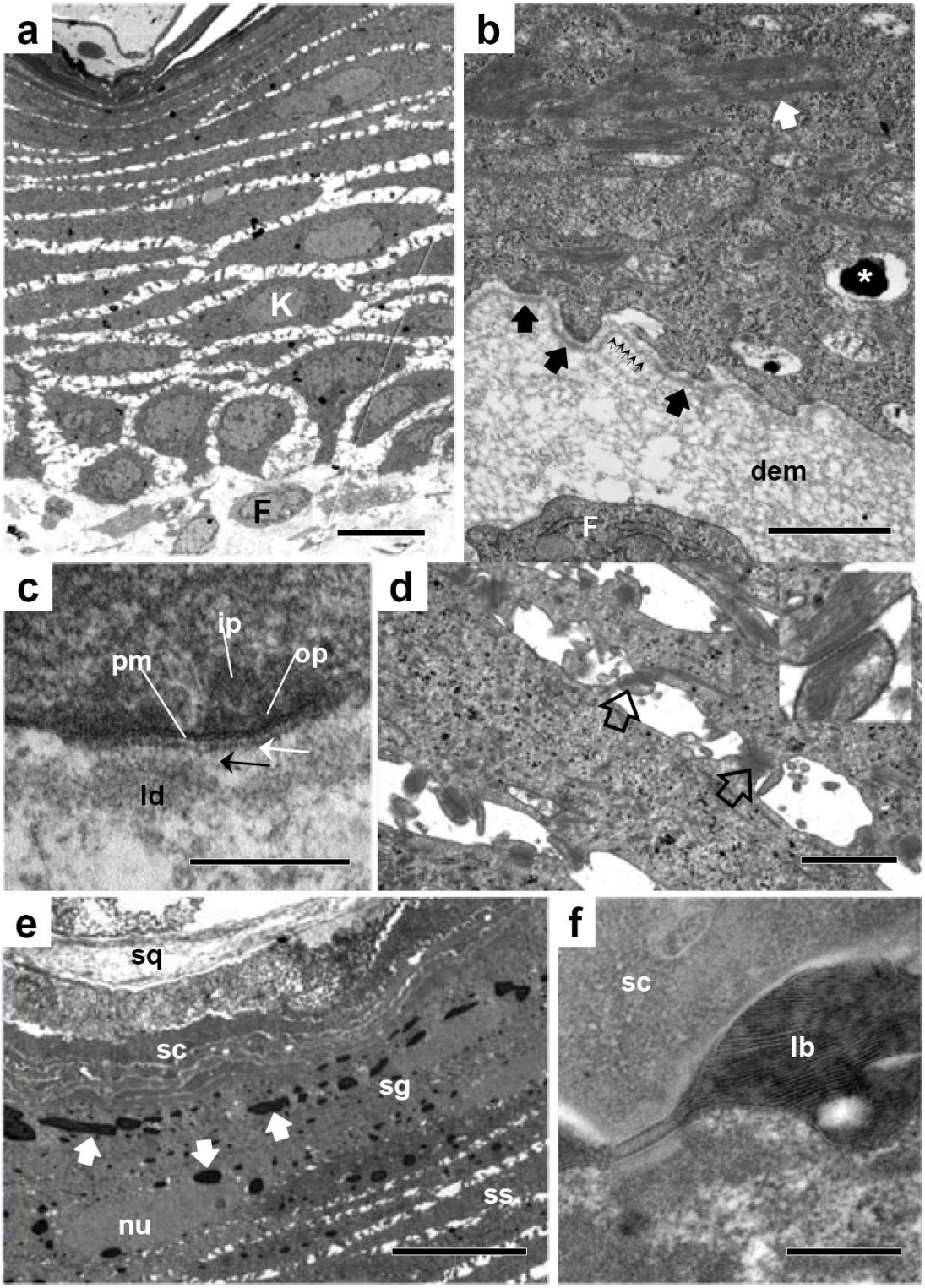

Figure 6. Transmission electron microscopy of a SdES after transplantation reveals that sweat gland-derived cells develop into characteristic human epidermal structures.

(**a**) The typical epidermal stratification and the characteristic adhesion of epidermal keratinocytes by numerous desmosomes are obvious. The lower pale cells are dermal fibroblasts (F). Dark cells are sweat gland-derived cells (S). (**b**) A mature

dermo-epidermal junction has developed. A basal lamina (group of small arrows) and hemidesmosomes (black filled arrows) as well as the keratin filament network (white single arrow) and an autophagosom (asterisk) in an epithelial cell are indicated. (dem = dermal extracellular matrix, F = fibroblast) (**c**) The complex structure of a hemidesmosome is shown (pm = plasma membrane, ip = inner plate, op = outer plate). A complete basal lamina is deposited consisting of the sublamina dense plate (white arrow), the lamina lucida (black arrow) and the lamina densa (ld). (**d**) Mature desmosomal connections (black arrows) between adjacent epithelial cells of the spinous layer. The inset depicts a typical desmosome at a higher magnification. (**e**) Magnification of the upper epidermal layers showing the stratum spinosum (ss), the stratum granulosum (sg) containing the dark keratohyalin granula (white arrows), the stratum corneum (sc) and skin squames (sq). (**f**) A lamellar body (lb) between two corneocytes in the stratum corneum (sc) is depicted. Scale bars: 10 µm (a, e), 5 µm (b), 1 µm (d) and 0.2 um (c, f).

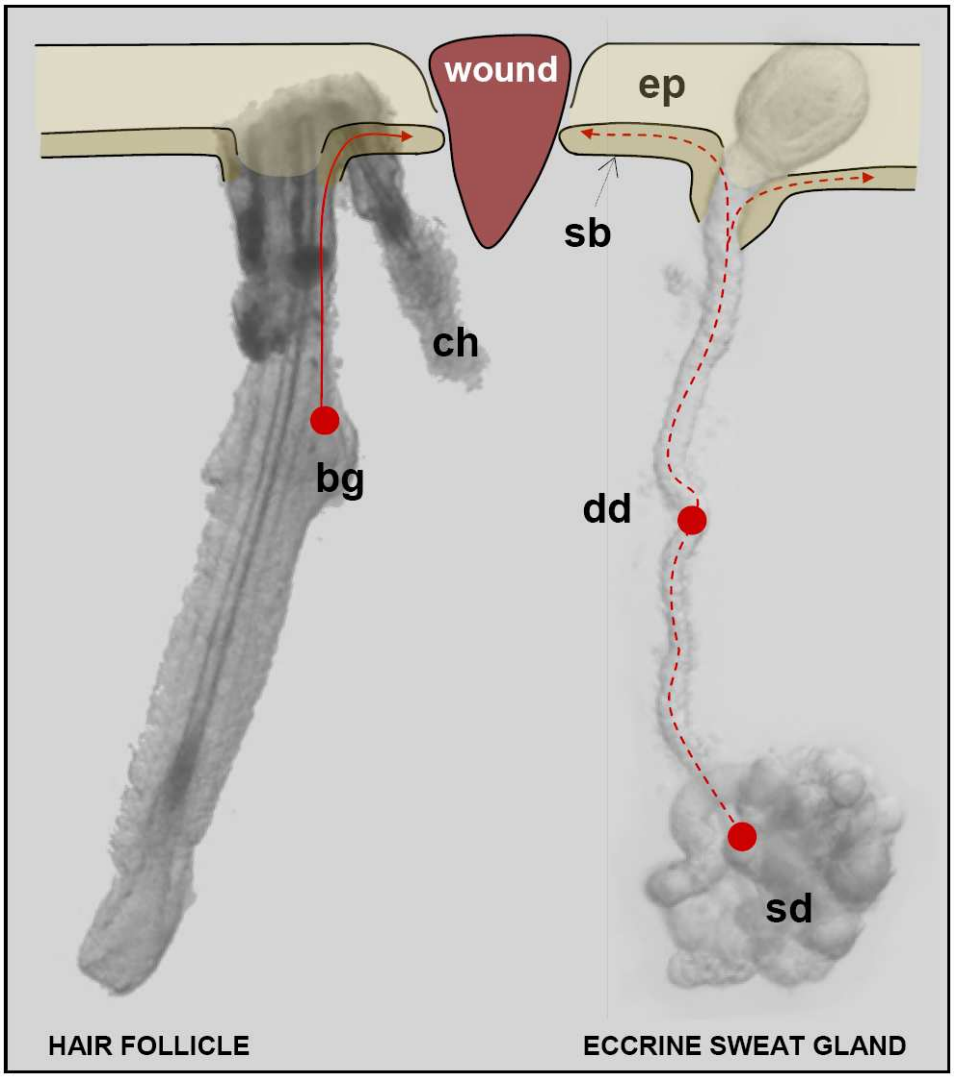

Figure 7. Hypothetical scheme considering both, the human eccrine sweat gland and the human hair follicle as sources for epidermal keratinocyte (stem) cells.

The human hair follicle bulge (bg) gives rise to epidermal keratinocytes that migrate towards the epidermis as it is locally injured upon wounding (ch = club hair). As eccrine sweat gland cells have the general potential to generate an epidermis (ep), sweat gland cells derived from the secretory domain (sd) or ductal domain (dd), may also migrate towards the epidermal stratum basale (sb) to participate in closing an epidermal wound. It may even be considered that sweat gland cells are involved in the homeostatic maintenance of the human epidermis.

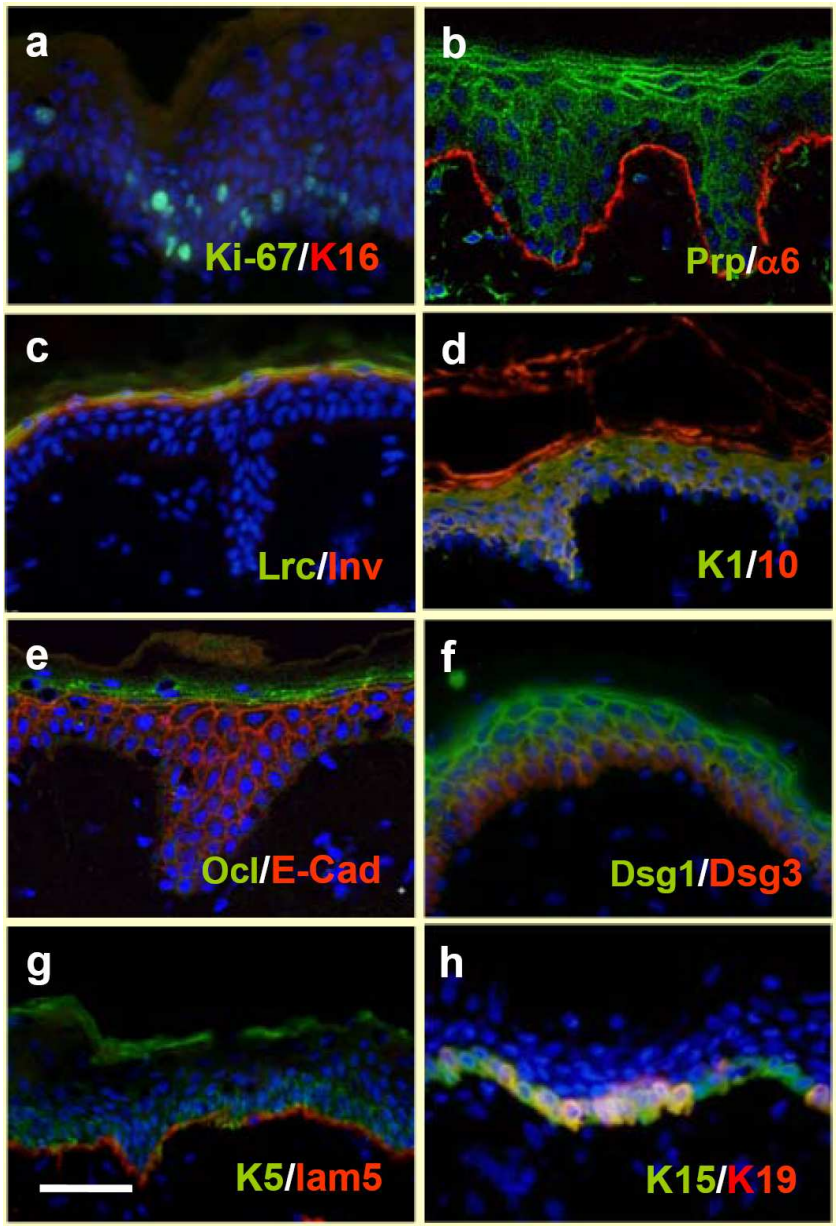

Figure S1. Distinct markers in normal human skin.

Indirect immuno-fluorescence of normal human skin using antibodies to (**a**) Ki-67/Cytokeratin 16, (**b**) Periplakin/Integrin-α6, (**c**) Loricrin/Involucrin (**d**) Cytokeratin 1/Cytokeratin 10, (**e**) Occludin/E-cadherin, (**f**) Desmoglein1/Desmoglein3, (**g**) Cytokeratin 5/Laminin 5 (Laminin-332) and (**h**) Cytokeratin 15/Cytokeratin 19. These antibodies were employed to create a normal reference for the analysis of our in vitro and vivo skin substitutes. Scale bar for all panels: 50 µm.

2.2 Markers to Evaluate the Quality and Self-Renewing Potential of Engineered Human Skin Substitutes In Vitro and after Transplantation

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Abstract

We screened a series of antibodies for their exclusive binding to the human hair follicle bulge. In a second step these antibodies were to be used to identify basal keratinocytes and potential epithelial stem cells in the human epidermis and in engineered skin substitutes. Of all the antibodies screened, we identified only one, designated C8/144B, that exclusively recognized the hair follicle bulge. However, C8/144B-binding cells were never detected in the human epidermal stratum basale. In the bulge C8/144B-binding cells gave rise to cytokeratin 19-positive cells, which were also tracked in the outer root sheath between bulge and the hair follicle matrix. Remarkably, cytokeratin 19-expressing cells were never detected in the hair follicle infundibulum. Yet, cytokeratin 19-expressing keratinocytes were found in the epidermal stratum basale of normal skin as a subpopulation of cytokeratin 15-positive (not C8/144B-positive) basal keratinocytes. Cytokeratin 19/cytokeratin 15-positive keratinocytes decreased significantly with age. We suggest that cytokeratin 19-expressing cells represent a subpopulation of basal keratinocytes in neonates and young children (up to 1.5 years) that is particularly adapted to the lateral expansion of growing skin. Our data show that cytokeratin 19 in combination with cytokeratin 15 is an important marker to routinely monitor epidermal homeostasis and (at least indirectly) the self-renewing potential of engineered skin.

Introduction

Epidermal self-renewal in native skin indispensably requires adult epidermal stem cells (SCs). This is equally true for all sorts of surgically harvested as well as laboratory grown skin grafts. Epidermal SCs are thought to reside in the basal layer of the interfollicular epidermis and are supposed to be unipotent SCs that give rise to only one cell lineage, namely keratinocytes (Brouard and Barrandon, 2003). Furthermore, there is convincing evidence that multipotent epithelial SCs are located in the bulge region of the hair follicle (HF; Blanpain et al., 2004; Morris et al., 2004). These cells have the potential of generating at least three different cell lineages including hair matrix cells, sebaceous gland cells, and epidermal keratinocytes (Alonso and Fuchs, 2003).

Although significant advances were made in identifying and locating the epithelial stem cell compartment in rodent skin (Cotsarelis et al., 1990; Taylor et al., 2000; Oshima et al., 2001; Braun et al., 2003; Blanpain et al., 2004; Fuchs et al., 2004; Morris et al., 2004), stem cell data regarding human skin are still vague. A number of putative stem cell markers, such as β1-integrin (Jones and Watt, 1993; Jones et al., 1995), α6-integrin in combination with CD71 (Li et al., 1998; Webb et al., 2004), CD34 (Trempus et al., 2003; Blanpain et al., 2004), AC133 (Belicchi et al., 2004), p63 (Pellegrini et al., 2001; Koster and Roop, 2004), keratin (K)15 (Lyle et al., 1998), ABCG2 (Terunuma et al., 2003; Triel et al., 2004), and BMI-1 (Park et al., 2003) have been suggested. However, convincing evidence that these markers exclusively identify cells that have the capacity to self-renew and to maintain long-term tissue integrity and function is mostly missing.

Clinical follow-up studies demonstrate that epidermis generated from cultured epidermal autografts can self-renew for decades (Carsin et al., 2000), indicating that a sufficient number of viable stem cells was initially present and survived in the transplanted graft. On the other hand there is also evidence that freshly transplanted cultured epidermal autografts are lost, due to the melting graft phenomenon, when grafts contain an insufficient number of viable epidermal stem cells (Matsumura et al., 1998).

According to the literature there are different views on how keratinocyte stem cells are organized in human skin. They may be arranged as epidermal proliferative units (Mackenzie, 1970; Potten and Morris, 1988), or be derived from skin appendages. Also, homeostatic regulation may cause suprabasal (K10 expressing) keratinocytes

to retrodifferentiate into unipotent self-renewing keratinocytes (Li et al., 2004), for instance in a wound with a shortage of selfrenewing keratinocytes.

On the basis of the above considerations we aimed at searching for tools suited to evaluate the quality and selfrenewing potential of engineered human dermoepidermal skin grafts. We present data showing that the C8/144B antibody is the only one in our antibody screening that recognizes a distinct cell population in the HF bulge. This cell population gives rise to K19-positive cells which may produce matrix cells that contribute to hair growth. We also provide evidence that K19-expressing cells represent a subpopulation of keratinocytes in the human stratum basale during proliferative lateral skin expansion. We have developed a one-step dermoepidermal transplantation model which reveals that K19 is a valuable marker to monitor the quality, homeostasis, and self-renewing capacity of engineered skin substitutes. Notably, we found that the situation in human skin differs in many aspects from that in mouse.

Results

Two types of epithelial cells can be distinguished in the hair follicle bulge of normal human skin

We have analyzed skin biopsies from human individuals (aged 1 day to 49 years). We intended to identify antibodies specifically binding to the human HF bulge, a region known to contain multipotent skin epithelial stem cells (Cotsarelis et al., 1990; Taylor et al., 2000; Oshima et al., 2001; Braun et al., 2003; Fuchs et al., 2004). In a second step we employed selected bulge-specific antibodies to search for keratinocyte stem cells within the stratum basale. Antibodies directed to the following antigens were tested: β1-integrin, α6-integrin, CD71, ABCG2, p63, Ki67, K15, antigen detected by C8/144B, K19, CD34, melanoma chondroitin sulphate proteoglycan, PLZF, BMI-1, CD200, follistatin, Dkk3, and Wif-1 (Table 1).

The only antibody, which exclusively bound to the HF bulge, was clone C8/144B from Dako (Baar, Switzerland; Figure 1a; Table 1). The C8/144B monoclonal antibody was originally generated against a short intracytoplasmic peptide of CD8. There are reports claiming that C8/144B recognizes K15 in the bulge region of human HFs (Lyle et al., 1998). Using confocal microscopy we confirmed that C8/144B indeed specifically binds to the HF bulge (Figure 1b and c; Table 1). Thus, we consider C8/144B the most reliable human HF bulge marker presently available.

The human HF bulge was also recognized by an antibody to K19, however, K19-positive cells were also detected along the variable region of the HF down to the HF matrix (Figure 1a). Importantly, K19-positive cells were never seen in the infundibulum above the bulge (Figure 1a), suggesting that HF-derived, K19-expressing cells do usually not give rise to epidermal cells (under nontraumatic conditions).

K19-positive cells originate from CD8/144B-expressing cells in the hair follicle bulge of normal human skin

The data shown in Figure 1a raise the possibility that K19-positive cells originate in the HF bulge and migrate from there to the HF matrix. Therefore, we also performed double immunofluorescence using C8/144B and K19 antibodies and confocal microscopy on HF bulge sections (Figure 1c–e).

We identified a region in the asymmetrically organized bulge, which consisted almost exclusively of cells recognized by the C8/144B antibody (Figure 1b). As the optical

sections approached the region underneath the C8/144B-positive cells (toward the centrally located hair shaft), a short zone of cells was detected by both the C8/144B (FITC conjugated) and the anti-K19 (PE conjugated) antibodies resulting in a yellow fluorescence (Figure 1c and d). Towards the inner cell layers of the bulge, cells recognized by C8/144B became rare, whereas K19-expressing cells were more abundant (Figure 1e). These data imply that C8/144B-binding and K19- expressing cells are organized in distinct layers in the human HF bulge. The existence of a small transitional zone in which both markers are expressed in the same cells suggests that K19-positive cells are directly derived from C8/144B-binding cells.

A scheme summarizing these results is shown in Figure 1f. In accordance to other published data, our findings suggest that there are two distinct populations of epithelial cells in the human HF bulge (Blanpain et al., 2004). However, in contrast to Blanpain et al., who described a CD34-positive cell population restricted to the mouse HF bulge, we detected CD34-expressing cells all along the outer root sheath in the human HF.

C8/144B does not recognize interfollicular keratinocytes

Upon testing the C8/144B antibody no cells were recognized in human interfollicular epidermis. This was invariably the case in human skin derived from different sites, such as foreskin, scalp, abdomen, back, and retroauricular (Table 2). This finding suggests that (multipotent) stem cells in the HF bulge are distinct from (unipotent) stem cells located in the epidermis.

To gain more insight into their K15 specificity, we compared C8/144B with two commercially available, K15-specific antibodies, designated LHK15 and SPM190. Proteins were extracted from HaCaT cells, which are known to express K15 on cell culture plastic (Werner and Munz, 2000) and analyzed in western blots. The LHK15 antibody clearly recognized a band of 55 kDa (Figure 2). SPM190 also showed the same prominent band, however, also weakly detected some additional proteins. Interestingly, C8/144B also showed weak binding to the diagnostic protein (Figure 2). Differences in the affinity of the three antibodies to K15 may account for these variations in staining intensities.

K19-positive cells are a subpopulation of K15-expressing keratinocytes in the interfollicular epidermis of normal human skin

In very young skin (from neonate to 1.5-year old) K15 was expressed in all keratinocytes of the stratum basale (Figure 3a), whereas in the skin of older patients it was expressed in cells of the lower parts of the rete ridges only (Figure 3b). Suprabasal keratinocytes did not show K15 expression. These data are in accord with previous studies (Waseem et al., 1999; Ghali et al., 2004; Porter et al, 2000; Webb et al., 2004) and imply that in human interfollicular epidermis K15 is not necessarily a stem cell marker but rather a marker for basal keratinocytes anchored to a functional basement membrane.

K19-positive basal keratinocytes were found in all body sites investigated (Table 2). Importantly, K19 was expressed in an age-dependent manner. The number of K19-positive basal cells accounted for 50–70% of basal keratinocytes in newborn skin (Figure 3c), 10–30% in infant skin (8 days to 2 years), and was hardly detectable in adult skin (18–49 years; data not shown).

K19-positive cells represented a subpopulation of K15- expressing basal keratinocytes (Figure 3d). K19/K15-expressing basal keratinocytes were almost always arranged in clusters (Figure 3d). K20 staining demonstrated that about 0.1% of all K19-expressing cells were Merkel cells (data not shown).

Basal keratinocytes-expressing K19 are indicators of an intact epidermal homeostasis in dermoepidermal substitutes engineered in vitro

To evaluate the fate of K19-expressing cells on a plastic substrate in culture, epidermal keratinocytes derived from children and adults (Table 2) were immunostained with K19 antibodies. At 2 days after plating, keratinocytes were completely spread out on the substrate. Several K19-positive cells were arranged in pairs (Figure 4a) indicating that the first mitoses had occurred. After 4 days in culture, K19-positive cells were still arranged in colonies (Figure 4b). After 7–10 days, the keratinocytes had become confluent in culture and about 30–50% of the cells expressed K19 (Figure 4c). The significant increase of K19-expressing keratinocytes derived from young children (up to 5 years) is shown in Figure 4d. In contrast, adult keratinocytes never gave rise to this high numbers of K19-expressing cells. In keratinocyte cultures derived from 40 to 50 years old patients we found 3–5% of K19-posive cells 7, 9, and 15 days after plating (Figure 4d). These data suggest that the

in vitro outgrowth of K19-positive keratinocytes decrease with increasing age. They also underscore the value of K19 expression as a marker to monitor the self-renewing and regenerating potential of a given epidermal substitute.

To determine the epidermal regenerative capacity of keratinocytes, primary cells were grown into multilayered epidermal constructs. Keratinocytes were plated onto collagen type-I hydrogels populated by dermal fibroblasts on which they developed into multilayered epidermal equivalents within 3 weeks (Figure 5a). These exhibited a basal layer of densely packed cells, followed by 10–15 layers of differentiating keratinocytes and some layers of terminally differentiated cells forming a stratum corneum (Figure 5a). Upon organotypic dermoepidermal culture on collagen type-I hydrogels, homeostatic regulation caused excess K19-positive keratinocytes (previously expanded on plastic) to be eliminated by their release into terminally differentiated strata. However, a substantial number of K19-expressing cells remained in the basal layer (Figure 5b). A basal lamina-like structure had been deposited, as demonstrated by antibodies to laminin 10 (Figure 5c). Furthermore, K15 was not expressed in the basal cells of these constructs (data not shown), indicating that the in vitro grown epidermis still lacks some significant organizational properties in vitro. However, as these substitutes perfectly survived after transplantation onto immunoincompetent nude rats, it is clear that (although K15 was not expressed in these basal keratinocytes) a selfrenewing keratinocyte compartment was existent. Ki67-expressing, that is proliferating keratinocytes, were present 4 weeks after seeding and were located almost exclusively basally (Figure 5d). In contrast to the situation on cell culture plastic, only a small percentage (3–5%) of K19-expressing cells was positive for Ki67, indicating cell proliferation (see also Figure 6h).

These findings are consistent with the view that K19- positive keratinocytes in the stratum basale reveal a ‘‘young’’ epidermis, and are indicators of intact tissue homeostasis in engineered skin substitutes.

Basal K15/K19-expressing cells are indicators of epidermal homeostasis in skin resulting from grafted in vitro engineered dermoepidermal substitutes

We sought to determine the regenerative potential and homeostasis of dermoepidermal composites after transplantation. Full thickness skin defects were created on immunoincompetent Nu/Nu rats and sheltered against the surrounding

skin by implanting a transplantation chamber, a modified Fusenig chamber (Fusenig et al., 1983), to prevent wound healing through rat-derived cells (Figure 6a).

The only dermoepidermal skin grafts that were rapidly and sufficiently vascularized after transplantation, and hence readily integrated into the wound, were those based on collagen type-I hydrogels. Importantly, these grafts could be transplanted in only one single surgical intervention. Histological analyses 21 days after transplantation revealed an epidermis with a near to normal stratification and discernable rete ridges (Figure 6b). The human origin of the transplanted keratinocytes was confirmed using a mouse antihuman nuclei monoclonal antibody (Figure 6c). K19-positive cells were relatively abundant and formed monolayered clusters, firmly attached to a basement membrane-like structure, as demonstrated by K19/laminin 5 double staining (Figure 6d). The number of K19-expressing cells, 3 weeks after transplantation was comparable to the number found in the skin of 0–2 years old children. Importantly, this was also true for grafts derived from keratinocytes of adult donors.

In contrast to nontransplanted skin substitutes, transplanted grafts exhibited a continuous layer of basal cells expressing K15, with K19-positive cells representing a subpopulation of these (Figure 6e). This is identical to the situation in native skin (Figure 3d).

Obviously, human dermal fibroblasts, initially submerged in polymerizing collagen type-I, remained viable and proliferated after transplantation, as a near normal dermal layer of human origin was clearly distinguishable from the underlying rat tissue using an antibody recognizing human fibroblasts, 3 weeks after transplantation (Figure 6f). Thus, dermoepidermal grafts after transplantation matured into tissues closely resembling normal human skin.

Co-staining the stratum basale with Ki67 and K19-specific antibodies and the subsequent quantification of doublepositive cells revealed that in human skin, in engineered grafts (in vitro), and in transplanted engineered substitutes (Figure 6g), the number of double-positive cells was about 5% of all K19-positive keratinocytes (Figure 6h). These findings are in accord with the hypothesis that the vast majority of K19-expressing keratinocytes are slowly proliferating (or nonproliferating). In contrast, 90% of the K19- expressing keratinocytes derived from young children (up to 5 years), when grown on cell culture plastic, became Ki67-positive and hence proliferating (Figure 6h). As a consequence these ‘‘young’’ (and initially quiescent) K19-positive cells were finally dominating the culture.

Comparing K19 expression with other potential keratinocytes stem cell markers

We were wondering whether K19-positive keratinocytes were also recognized by other described keratinocyte stem cell markers. In particular the combination of α6-integrin and CD71 (transferrin receptor) antibodies appears to be relevant in this respect. α6-Integrin-bright (bri)/CD71-dim keratinocytes have been published to be enriched for keratinocytes stem cells (Li et al., 1998; Tani et al., 2000). Our Fluorescence activated cell sorting (FACS) analyses revealed that the α6-integrin-bri/CD71-dim fraction shows indeed significantly more K19-positive keratinocytes (up to 3x more) than the initially prepared, total keratinocyte population (Figure 7). The α6-integrin-dim population contained almost no K19-positive cells (data not shown).

Discussion

The goal of this study was to identify markers suited to evaluate epidermal homeostasis and the self-renewing potential of skin substitutes, both in vitro and after transplantation. As the C8/144B antibody binds exclusively to the HF bulge, the corresponding cells are most likely identical to multipotent epithelial stem cells (Cotsarelis, 2006). We provide evidence that these cells give rise to two distinct cell fractions. One C8/144B-binding fraction remains in place to maintain the multipotent stem cell pool. The second fraction develops into a transiently existing cell population which binds both C8/144B and K19-specific antibodies. These double-positive cells then give rise to K19- expressing cells which are no longer recognized by C8/144B. According to the stem cell migration hypothesis (Fuchs et al., 2001; Oshima et al., 2001), K19-positive cells may represent unipotent, self-renewing keratinocytes exiting the bulge and migrating along the outer root sheet to the basis of the HF. Here they may maintain the pool of matrix cells that contribute to hair growth.

K19-expressing keratinocytes are not found in the constant region of the HF above the bulge. Hence, bulge-derived K19-positive cells may not contribute to epidermal renewal (under normal, homeostatic conditions). This view is supported by convincing recent reports showing that bulge-derived stem cells are not responsible for maintaining the interfollicular epidermis in homeostatic conditions (Claudinot et al., 2005; Ito et al., 2005; Levy et al., 2005).

Nevertheless, we found a distinct population of K19-positive keratinocytes in both the stratum basale of young individuals and the basal layer of engineered, stratified skin substitutes. The clustered pattern of these keratinocytes corresponds to the distribution one would expect for selfrenewing keratinocytes in the stratum basale. Concomitantly, there are several reports stating that K19-expressing keratinocytes represent self-renewing cells (Lane et al., 1991; Jones et al., 1995; Michel et al., 1996; Cotsarelis et al., 1999; Akiyama et al., 2000). On the basis of our data the following questions arise: What are the properties of the distinct set of K19-positve keratinocytes in the human stratum basale? Why are K19-expressing cells so dramatically reduced with age?

K19-positive keratinocytes are highly abundant in the epidermis of fetuses and neonates (Van Muijen et al., 1987; Kwaspen et al., 1997). We show here that K19-positive keratinocytes are still detectable in the stratum basale of 1.5-year-old children. The epidermis of these individuals is characterized by rapid lateral growth.

We suggest that K19-positive cells represent a population that is adapted to this rapid lateral expansion of the epidermis and represents a keratinocyte population that is uncommitted to terminal squamous differentiation. As with progressing age this lateral growth ceases, this type of cell is no longer required. Stratification and permanent regeneration (vertical maintenance), however, are ongoing in adult skin. This of course also requires self-renewing keratinocytes, which in adult skin are extremely rare or K19-negative. It still remains to be determined where in the adult, interfollicular self-renewing keratinocytes are derived from. As in the adult, significant numbers of K19-expressing cells are located in the HF, it cannot be excluded that these cells contribute to the basal epidermal cell pool, should there be a shortage of selfrenewing cells. An additional interesting possibility is that in humans keratinocyte stem cells may derive from sweat glands (which are distributed almost throughout the entire human skin), an issue that still is only moderately investigated.

How do K19-expressing human keratinocytes compare to K19-positive keratinocytes in mouse? The murine K19 gene shows high homology to its human counterpart, and the gene is located in the acidic keratin cluster on mouse chromosome 11 (Lussier et al., 1990). In both species precursor cells in different tissues display high K19 levels (Brembeck and Rustgi, 2000). However, in mouse K19 is expressed in the HF but is absent from the interfollicular epidermis at hairy sites (Michel et al., 1996). Once again this difference has to be taken into account when comparing biological phenomena in both species.

Conventional cell culture conditions provide an environment in which dissociated (single) primary keratinocytes have to newly establish their epithelial phenotype. The regulatory mechanisms of stratification and tissue homeostasis are greatly disturbed under these culture conditions. These conditions may represent a situation of extreme wound healing under which initially quiescent K19-expressing cells enter a state of proliferation. In contrast, organotypic (dermoepidermal) culture of keratinocytes, using collagen hydrogels that contain fibroblasts, induces epidermal stratification and tissue homeostasis. As a consequence surplus K19-positive cells (having accumulated during cell expansion on a plastic substrate) are now eliminated by terminal differentiation. Yet, a physiologically reasonable number of basal K19-expressing keratinocytes remains in the basal layer.

As for the quality control of engineered skin substitutes, these basal, K19-positive cells are important indicators of a young, proliferating, and self-renewing graft.

It has been reported that the C8/144B antibody recognizes K15 and defines the location of human HF stem cells in the bulge (Lyle et al., 1998). However, further work demonstrated that K15 expression is not only restricted to the bulge but is also expressed in a significant stretch of the outer root sheath of the human HF and in the stratum basale of human epidermis (Porter et al., 2000). We show here that the C8/144B antibody indeed weakly recognizes K15 in immunoblots, whereas it does not detect keratinocytes in the human stratum basale. A possible explanation for this discrepancy is that in human HF bulge cells, a distinct epitope of K15 is exposed, which is masked in the keratinocytes of the stratum basale. Furthermore, it is likely that there are differences in the affinities of the three K15-specific antibodies used in our immunostainings. Additional experiments are required to shed some more light on this issue.

Using collagen type-I hydrogels and a rat transplantation model we were able to achieve rapid vascularization and functional integration of complex dermoepidermal skin substitutes after one single surgical intervention. This was not possible using porous lyophilized collagen scaffolds (sponges) for epidermal reconstitution, because vascularization was too slow to keep the epidermal substitute alive.

It was also evident that transplantation of engineered dermoepidermal composites was a crucial step in completing physiological differentiation and epidermal stratification. Employing K19 and K15 as markers, it became possible to show that the organism is an extremely efficient bioreactor and a perfect regulator of organ structure and function.

In summary it can be said that K19/K15-double-positive keratinocytes represent a distinct basal-cell population in growing skin. For engineered skin substitutes, K19 and K15 in combination are valuable tools to monitor tissue homeostasis and the potential to self-renew.

Materials and Methods

Preparation of skin specimens

Human skin samples were taken from the scalp, the abdomen, the retroauricular region, or from foreskins. Parents or patients gave their written informed consent. The medical ethical committee of the Kanton Zurich approved all described studies. Furthermore this study was conducted according to the Declaration of Helsinki Principles. Tissues were embedded in OCT compound (Sakura Finetek/Digitana AG, Horgen, Switzerland) and placed in dry ice. Cryosections of 6–30 mm were cut at -30 ℃.

Isolation and culture of keratinocytes and fibroblasts

Skin biopsies were digested for 15–18 hours at 4 ℃ in 12 U ml^{-1} dispase in Hank's buffered salt solution containing 5 µg ml^{-1} gentamycin. Thereafter the epidermis and the dermis were separated using forceps. The epidermis was further digested in 1% trypsin, 5mM EDTA for maximal 3 minutes at 37 ℃. T he dermal tissue was digested in 2 mg ml^{-1} collagenase for approximately 60 minutes at 37 ℃. Epidermal cells were resuspended in serum-free keratinocytes medium containing 25 µg ml^{-1} bovine pituitary extract, 0.2 ng ml^{-1} EGF, and 5 µg ml^{-1} gentamycin. A total of $4x10^6$ dermal cells per Ø10cm dish were grown in DMEM supplemented with 10% fetal calf serum, 4mM L-alanyl-L-glutamine, 1mM sodium pyruvate, and 5 µg ml^{-1} gentamycin. Collagenase was from Sigma (Buchs, Switzerland), all other compounds were from Invitrogen (Basel, Switzerland).

Organotypic cultures

Organotypic cultures were prepared using a previously established transwell system (six-well cell culture inserts with membranes of 3.0 mm pore size; BD Falcon, Basel, Switzerland). The membranes were covered with collagen type-I hydrogels which contained $1x10^5$ human dermal fibroblasts (passage 1). These dermal equivalents were grown in DMEM for 6 days to allow for gel contraction. Subsequently $5x10^5$ basal keratinocytes were seeded on each dermal equivalent. Triplicate wells were set up for each dermoepidermal substitute. Keratinocytes were cultured for 4 days in three parts of DMEM and one part of Ham's F12, 0.3% fetal calf serum, 4mM L-glutamine, 1mM sodium pyruvate, 5 µg ml^{-1} gentamycin (all; Invitrogen), 0.4 µg ml^{-1}

hydrocortisone, 5 µg ml^{-1} insulin, 5 µg ml^{-1} transferrin, 2nM triiodothyronine, 180 µM adenine, 5.3 pM sodium selenite, 20 nM progesterone, and 1.8mM CaCl2 (all; Sigma). After 4 days the keratinocyte layer was raised to the air/liquid interface and cultured for 3 additional weeks. During the first week, the culture medium was a 1:1 mix of DMEM and Ham's F12 containing the supplements described above and 2% FCS (Invitrogen). The fetal calf serum was reduced to 1% during the second and third week. Cultures were finally processed for transplantation or for cryo- and paraffin sections.

Transplantation of cultured dermoepidermal composites

Dermoepidermal grafts were transplanted onto full thickness skin defects created surgically and encased by polypropylene rings, 27mm in diameter (modified Fusenig chamber; Fusenig et al., 1983). The rings were sutured on the back of 10-week-old, female athymic Nu/Nu rats. The transplants were covered with a silicon foil. After 14 days the grafts were excised in toto and processed for cryo- and paraffin sections. Anesthesia for all procedures was performed using isoflurane (Abbott AG, Baar, Switzerland).

Antibodies

CD8 (clone C8/144B, 200 µg ml^{-1}, 1:30) from Dako (Switzerland AG, Baar, Switzerland); CD71 (clone berT9, 1:10): K19 (clone RCK108, 1:100); K20 (clone Ks20.8, 1:100); K10 (clone DE-K10, 1:100) from Santa Cruz (Labforce AG, Nunningen, Switzerland); laminin 5 (clone P3H9-2, 1:100); p63 (clone 4A4, 1:100); Dkk3 (polyclonal, 1:100); Wif-1 (polyclonal, 1:100) from Chemicon (Millipore AG, Zug, Switzerland); K15 (clone LHK15, 100 µg ml^{-1}, 1:100; clone SPM190, 100 µg ml^{-1}, 1:50); human nuclei (clone 235-1, 1:50); CD49f (clone 4F10, 1:200) from R&D Systems (Abingdon, UK); ABCG2 (clone 5DS, 1:20); follistatin (clone 85918, 1:40); melanoma chondroitin sulphate proteoglycan (clone LHM-2, 1:500) from BD Pharmingen (Basel, Switzerland); Ki67 (clone B56, 1:200); CD34, PE conjugated (clone 581, 1:50) from ABD Serotec (Dusseldorf, Germany); CD200 (clone MRC OX104, 1:20) from Dianova (Hamburg, Germany); human fibroblast (clone AS02, anti-CD90/Thy-1, 1:100) from Calbiochem (VWR, Dietikon, Switzerland); PLZF (clone 2A9, 1:50) from Upstate (Millipore AG); BMI-1 (clone 22F6, 1:100) from Spring Bioscience (AMS Biotechnology, Bioggio, Switzerland). Goat anti-mouse coupled

with horseradish peroxidase (polyclonal). For double immunofluorescence, some of the primary antibodies were prelabeled with Alexa 555-conjugated polyclonal goat F(ab0)2 fragments, according to the instructions of the manufacturer (Zenon Mouse IgG Labeling Kit; Molecular Probes/Invitrogen, Basel, Switzerland).

Immunohistochemical staining

Sections and/or cells were fixed and permeabilized in acetone for 5 minutes at -20 ℃, air dried and washed 3x in phosphate-buffered saline (PBS). Thereafter they were blocked in PBS containing 2% BSA (Sigma) for 30 minutes. Incubation with the diluted first antibody was performed in blocking buffer for 1 hour at room temperature. Slides were washed three times for 5 minutes in PBS and blocked for additional 15 minutes. The secondary antibody was added for 1 hour. Thereafter sections and/or cells were incubated for 5 minutes in PBS containing 1 µg ml^{-1} Hoechst 33342 (Sigma) and then washed twice for 5 minutes in PBS. Finally, the probes were mounted with Dako mounting solution (Dako) containing 25 mg ml^{-1} of DABCO anti-quenching agent (Sigma).

Western blotting

HaCaT cells ($3x10^6$) were lysed on ice in 1ml high salt lysis buffer (1.5 M KCl, 0.5% Triton X-100, 5mM EDTA) containing a proteinase inhibitor cocktail (Roche Diagnostics AG, Rotkreuz, Switzerland). The lysate was centrifuged at 15,000g at 4 ℃ for 10 minutes and the pellet was solubilized in 200 ml 9M urea, 50mM Tris-HCl pH 7.5 for 20 minutes at room temperature. Twenty ml of 6x Lämmli Loading buffer containing β-mercaptoethanol was added to 100 µl of the extract, and boiled for 5 minutes. Aliquots (25 µl) of the sample were loaded per lane and SDS–PAGE was performed. Semi-dry western blotting was done according to standard protocols. After blocking (blocking reagent; Roche), the membranes were incubated overnight with primary antibodies, diluted 1:100 in blocking reagent. After three washes in Tris-buffered saline Tween-20, the membranes were incubated for 1 hour with goat anti-mouse antibodies coupled with horseradish peroxidase, diluted 1:1000 in blocking reagent, followed by three washes in Tris-buffered saline Tween-20. Detection was performed using the ECL Plus kit (GE Healthcare Europe GmBH, Otelfingen, Switzerland) and a ChemiDoc-It imaging station (UVP).

Fluorescence microscopy

Fluorescence microscopy was performed using a Nikon Eclipse TE2000-U inverted microscope equipped with Hoechst, FITC, and TRITC filter sets (Nikon AG, Egg, Switzerland). For confocal imaging, the Eclipse TE2000-U was upgraded with a Nikon C1 Laser Scanning Microscope. A helium–neon laser with 543nm excitation was used for tetramethyl rhodamine iso-thiocyanate and an argon laser with 488nm excitation was used for FITC. With the Plan Apo x40 c/N.A. 0.95 objective 50 optical sections with an increment of 0.5 mm were captured. The line average was set to 4. Images were processed with Photoshop 7.0 (Adobe Systems Inc., Munich, Germany).

CONFLICT OF INTEREST

The authors state no conflict of interest.

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Table 1. Presumptive keratinocyte stem cell-specific antibodies

			Binding in human HF		Binding in human epidermis	
Antigen	**Reference**	**Antibody (clone)**	**Bulge**	**ORS/IRS**	**Basal layer**	**Suprabasal**
CD34	Blanpain *et al.* (2004)	581, My10, 8G12	+	+	–	–
CK19	Michel *et al.* (1996)	RCK108	+	+	+	–
CK15	Ohyama *et al.* (2006)	LHK15, SPM190	+	+	+	–
CK15	Lyle *et al.* (1998)	C8/144B	+	–	–	–
p63	Koster and Roop (2004)	4A4	+	+	+	+
Dkk3	Ohyama *et al.* (2006)	Polyclonal	–	–	–	–
Wif-1	Ohyama *et al.* (2006)	Polyclonal	–	–	–	–
MCSP	Legg *et al.* (2003)	LHM-2	+	+	+	–
CD133	Belicchi *et al.* (2004)	AC133	+	+	+	–
Follistatin	Ohyama *et al.* (2006)	85918	+	+	+	–
β1-Integrin	Jones and Watt (1993)	7F10	+	–	+	+
PLZF	Costoya *et al.* (2004)	2A9	+	+	–	+
BMI-1	Park *et al.* (2003)	22F6	+	+	+	+
ABCG2	Triel *et al.* (2004)	5DS	–	+	+	+

ORS/IRS, outer root sheath/inner root sheath
The presumptive keratinocyte stem cell-specific antibodies, tested on interfollicular epidermis and the hair follicle outer root sheath.

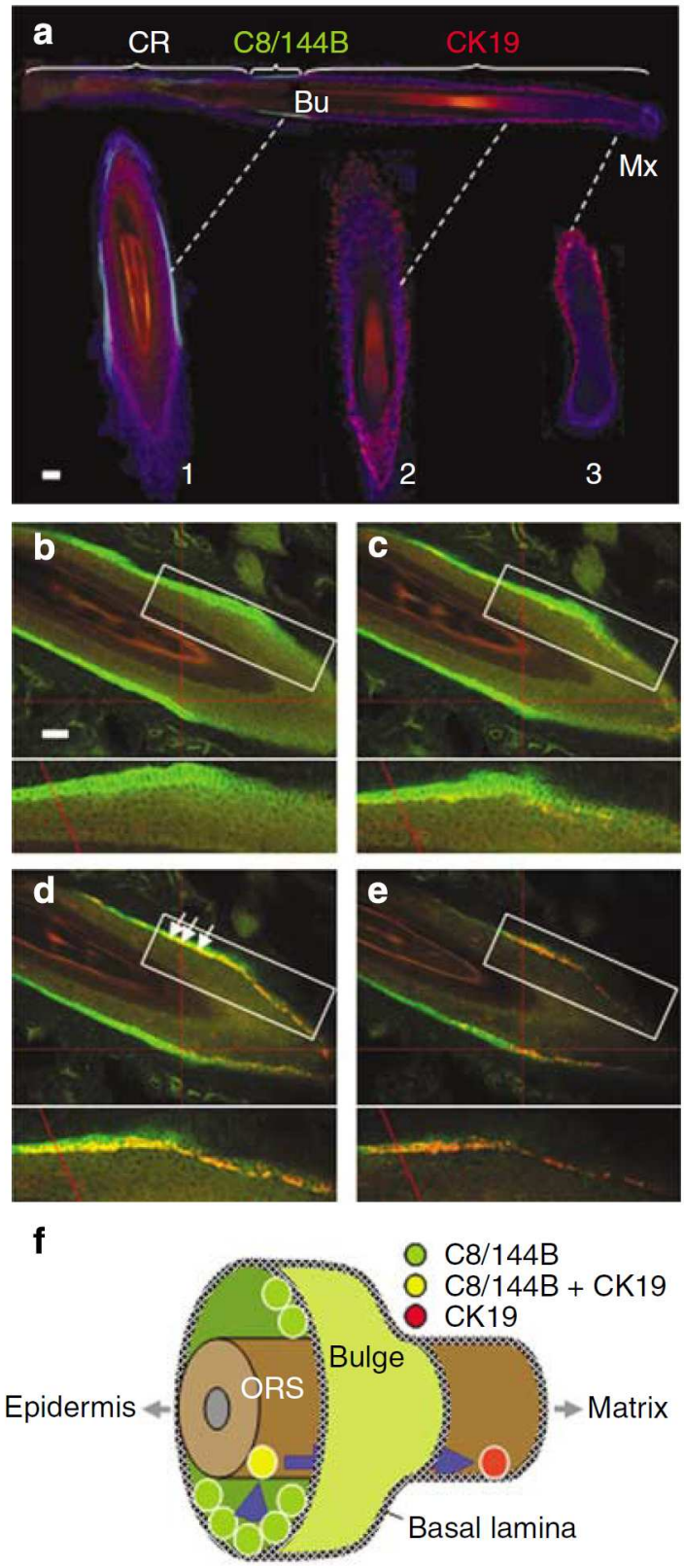

Figure 1. Two different cell types can be distinguished in the human hair follicle bulge. (a) Double immunofluorescence employing C8/144B (green) and anti-K19 (red) antibodies on scalp skin of a 3-year-old child. The positions of the three cross-sections through the hair follicle are indicated by 1, 2, and 3. C8/144B exclusively binds to cells of the HF bulge (Bu and 1). K19-specific antibodies

recognize cells in the bulge and all along the outer root sheath (2) down to the HF matrix (Mx and 3). Nuclei are counterstained with the Hoechst dye 33342 (blue). None of the two antibodies binds to the constant region (CR) above the HF bulge. (b–e) Serial confocal sections show the asymmetric organization of the HF bulge and reveal C8/144B-binding multipotent stem cells (green). Boxes (white frames) indicate the magnified region depicted underneath. (c, d) A thin layer of C8/144B and K19-doublepositive cells (arrows in d, and yellow cells in the magnified field in d) becomes obvious underneath the layer of C8/144B-positive cells. (e) Bulge-derived, K19-expressing cells at the lower end of the bulge region. (f) Scheme, summarizing the immunofluorescence patterns (Bu, bulge; CR, constant region; Mx, hair follicle matrix). All scale bars: 50 mm.

Table 2. K19 and K15 expression in basal keratinocytes in correlation with donor sites and donor age

Scalp			Abdomen		
Years	CK15	CK19		CK15	CK19
1.5	++	+	8 days	+++	+++
1.8	++	–	10 months	+++	+
2.2	++	–	17 years	++	–
6.3	++	–	18 years	++	–
6.3	++	–	41 years	++	–
7.5	++	–	49 years	++	–
14	++	–			
			Back		
				CK15	CK19
			1 days	+++	+++
Retroauricular			Foreskin		
	CK15	CK19	years	CK15	CK19
21 days	+++	+++	1	+++	++
11 years	++	–	6	++	–
12 years	++	–	15	++	–

K19 expression is detectable in skin of children not older than 2 years, whereas K15 is expressed in all sites, at all ages indicated.
+, 5–10% of basal keratinocytes.
++, 20–30% of basal keratinocytes.
+++, 50–70% of basal keratinocytes.
–, no basal keratinocytes recognized.

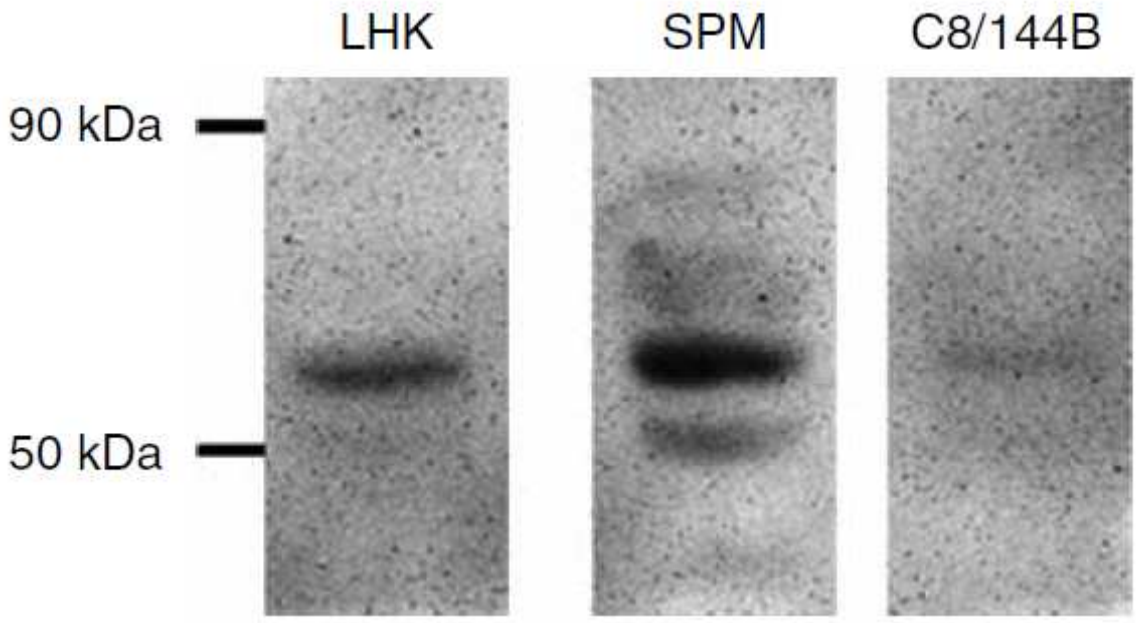

Figure 2. Testing different K15-specific antibodies by immunoblotting. Three different antibodies, LHK15, SPM190, and C8/144B were used. LHK15 and SPM190 clearly recognized a band of 55 kDa. C8/144B also showed some weak binding to the 55 kDa band. Equal volumes (25 ml) of the identical lysate were loaded.

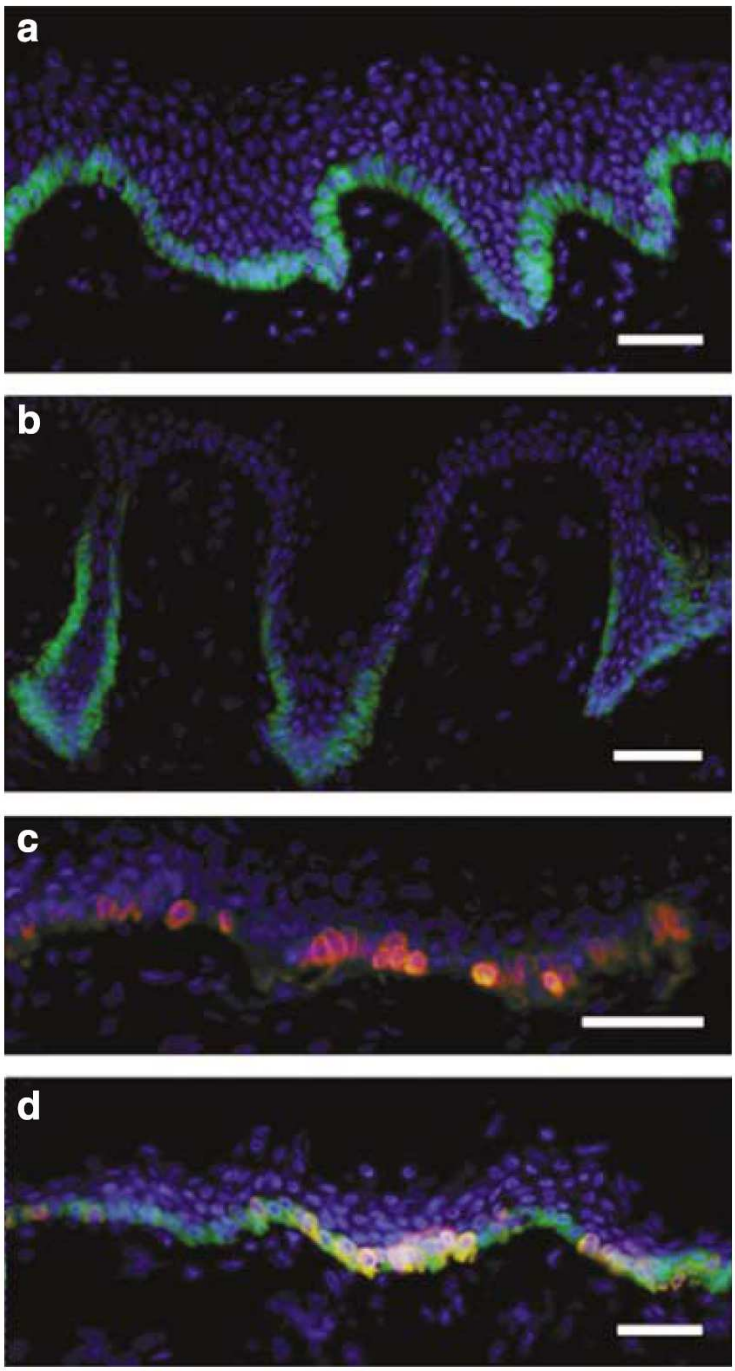

Figure 3. K19-expressing cells are a subpopulation of K15-positive keratinocytes. (a) All basal keratinocytes of the abdominal epidermis of a 10-month-old child express K15 (green). (b) Basal keratinocytes derived from the back of an 8-year-old child express K15 in the tips of the rete ridges. (c) About 50% of all basal keratinocytes isolated from the back of a 1-day-old neonate express K19 (red). (d) Double immunofluorescence using K15 (green) and K19 (red) antibodies on the epidermis derived from the retroauricular skin of a 3-week-old child. Note that K19-positive cells are a subpopulation of K15-expressing cells. Nuclei are counterstained with the Hoechst 33342 dye (blue). All scale bars: 50 mm.

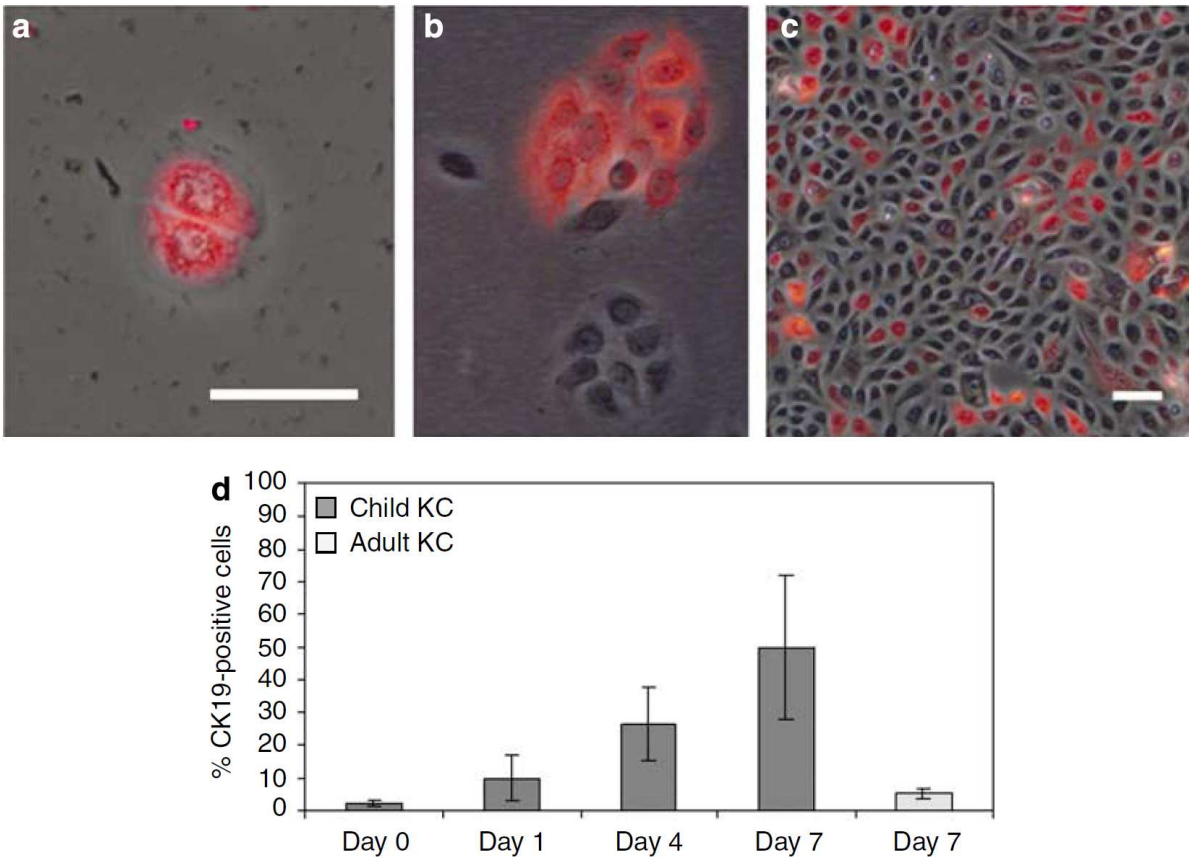

Figure 4. K19-positive keratinocytes proliferate under non-homeostatic conditions on cell culture plastic. (a) Doublets of K19-expressing keratinocytes. (b) K19-positive and K19-negative colonies of human keratinocytes can be observed 4 days after plating. (c) A confluent layer of keratinocytes 8 days after plating. A total of 30–50% of the cells express K19. (d) Quantification of K19-expressing cells isolated from children (from neonate to 5 years) at four different time points, reveals a steadily increasing number of K19-positive keratinocytes on cell culture plastic. Notably, adult (40 to 50-year old) keratinocytes show 10 times less K19-positive cells after 7 days in culture. Dark columns represent results with infant keratinocytes (infant KC). Bright columns represent results with adult keratinocytes (adult KC).

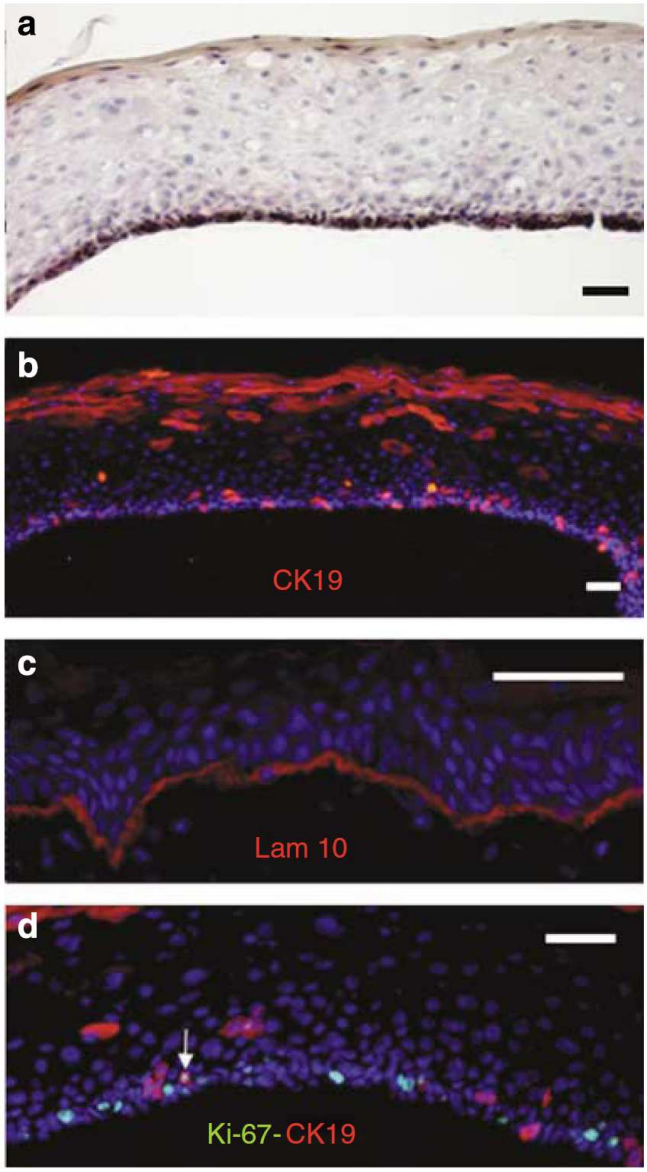

Figure 5. Evaluation of engineered epidermis equivalents. (a) Histological section and H/E staining of an in vitro engineered human epidermis equivalent grown on a collagen type-I gel which contains human dermal fibroblasts, 4 weeks after plating. The epidermis equivalent consists of a stratum basale, 10–15 keratinocyte layers, and a stratum corneum. (b) K19-expressing keratinocytes are apparently not yet polarized and are somewhat scattered in the basal layer. Homeostatic regulation in an organotypic graft causes excess K19-positive keratinocytes (created during their propagation on cell culture plastic) to be terminally differentiated, and hence eliminated, during stratification. (c) Laminin 10 staining shows that a basement membrane is about to be deposited. (d) Ki67/K19 double staining. Proliferating, Ki67-expressing, keratinocytes are located basally. The arrow indicates a rare Ki67/K19-double-positive keratinocyte. All scale bars: 50 mm.

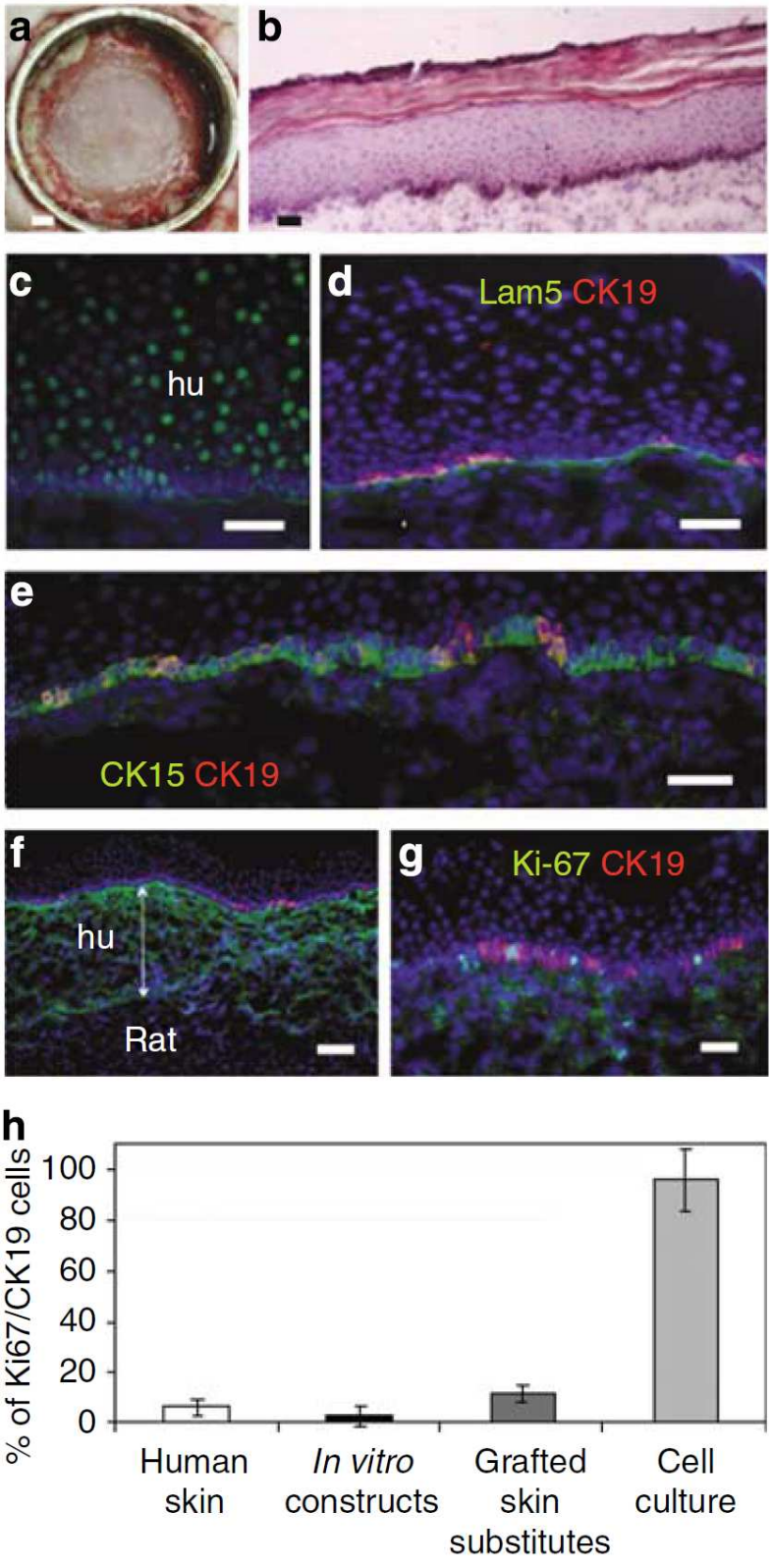

Figure 6. K19-positive human keratinocytes are indicative of an intact epidermal homeostasis in a grafted dermoepidermal skin substitute.

(a) Dermoepidermal skin substitutes consisting of an epidermal equivalent and a collagen type-I hydrogel containing dermal fibroblasts was transplanted on the back of immunoincompetent Nu/Nu rats. At 3 weeks after transplantation, an intact human

epidermis covered the former full thickness wound. (b) H/E staining of cryosections through the grafted area 3 weeks after transplantation shows a near normal skin histology apart from missing skin appendages. The epidermis reveals an almost normal stratification with already developing rete ridges. It is tightly connected to the underlying dermis. (c) The antibody specific to human nuclei confirms the human origin of the epidermis (green fluorescence). (d) K19-positive cells (red) are organized in clusters. Note that K19-positive cells are restricted to the well-defined stratum basale. These cells are strictly adhering to the continuous basement membrane (green) which here is visualized by a laminin-5 antibody. (e) In contrast to the nontransplanted engineered graft, K15 (green) is now expressed in all keratinocytes of the basal-cell layer. K19-expressing cells (red) are a subpopulation of K15-positive keratinocytes. (f) Human dermal fibroblasts (green) are recognized by the human CD90 (Thy-1)-specific antibody, whereas the rat tissue underneath stains negatively. (g) Within the clusters of K19-expressing cells only few, if any, cells were proliferating as shown by antibodies to the cell-cycle marker Ki67. (h) The vast majority of K19-expressing keratinocytes are slowly proliferating (or nonproliferating) as seen in human skin, in engineered constructs in vitro, and after their transplantation in vivo. In contrast, when dissociated and grown on cell culture plastic, almost every K19-expressing cell becomes Ki67-positive, thus indicating intensive cell proliferation. All scale bars: 50 mm.

	Total	R4 – R21 ($\alpha6^{bri}CD71^{bri}$)	R21 ($\alpha6^{bri}71^{dim}$)
CK19+	1%	21%	49%

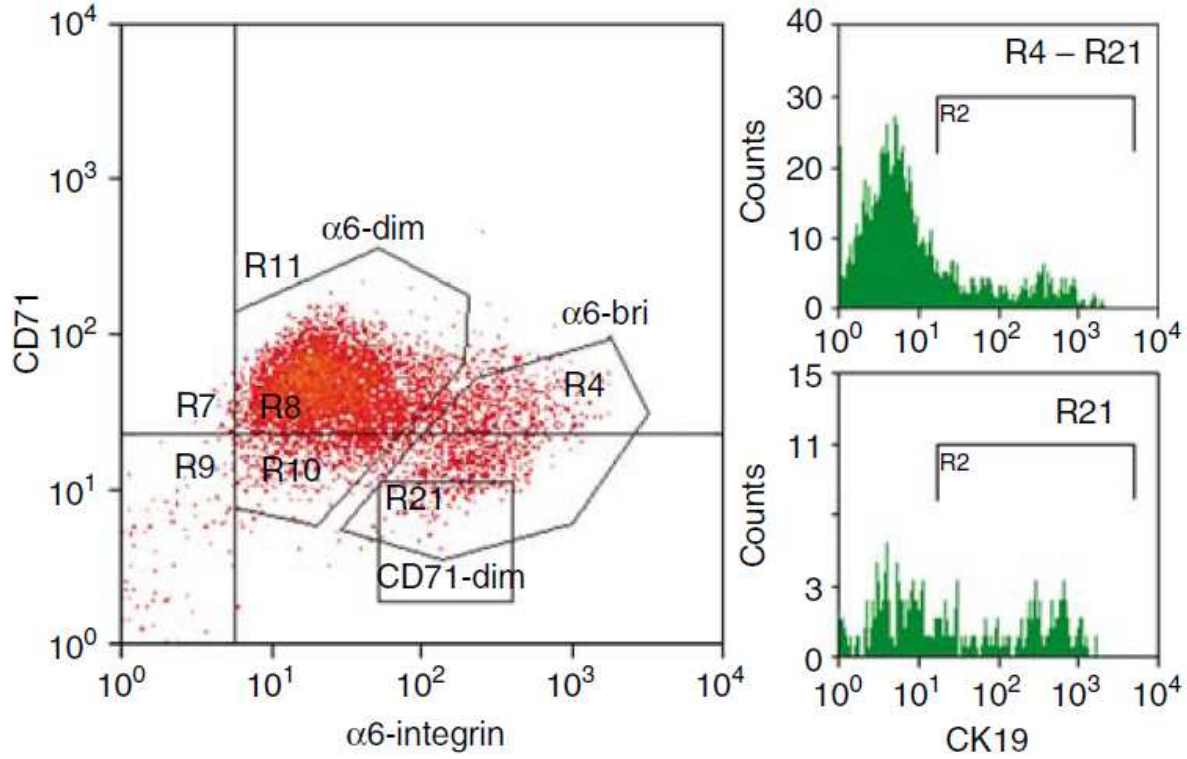

Figure 7. Comparing K19-positive keratinocytes and α6-integrin-bright/CD71-diminished cells. Staining primary keratinocytes for α6-integrin, results in two major cell populations, one of which shows low a6 expression (α6-dim), whereas the other exhibits high α6 expression (α6-bri). A subpopulation of α6-bri cells expresses low levels of CD71 (α6-bri/CD71-dim). α6-bri/CD71-dim cells are thought to contain keratinocyte stem cells. FACS analyses reveals that these α6-bri/CD71-dim fraction contains 49% K19-positive keratinocytes, whereas only 1% of the total keratinocytes preparation are K19 positive.

2.3 Matriderm versus Integra: A Comparative Experimental Study

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Abstract

Aim: To compare engraftment rates and vascularisation in a rat model using either Integra Artificial Skin® or Matriderm®.

Methods: Matriderm® and the dermal part of Integra® were compared in a two-step procedure including matrix implantation and subsequent epidermal grafting. Neonatal rat epidermis was used as coverage to test for rapid and complete take.

Results: Efficiency and quality of vascularisation expressed by take rate of epidermis, and thickness of resulting neodermis, were identical for both matrices.

Conclusion: This first comparison of Matriderm® with Integra® in a rat model revealed no major differences in engraftment rates or vascularisation.

Introduction

Since its introduction in the late 1980s [1], Integra® dermal regeneration template, an irreversibly cross-linked collagen/glycosaminoglycan matrix, has been widely used for dermal replacement in reconstructive surgery [2–6]. It has offered for the first time the possibility of building up two-layered skin after excision of full-thickness burns, scars, or giant naevi [7–9]. As it has some drawbacks, alternative matrices have been developed consisting of less densely packed collagen fibres. Matriderm®, a collagen/elastin matrix that is not chemically cross-linked, is one of the few alternatives that are licensed for reconstructive surgery [10–13]. Both Integra® and, to a lesser extent, Matriderm® have been studied in various settings [3,13], but a direct comparison of the two has never been performed. Thus, there is no information as to whether more or less densely packed collagen has superior clinical performance. The goal of this study was to compare Matriderm® and the dermal part of Integra® in an animal model, with particular focus on the time period needed for sufficient matrix vascularisation to allow epidermal graft take, and on the thickness and architecture of the neodermis.

Materials and Methods

Study design

Either Matriderm® or the dermal part of Integra® was implanted onto full-thickness skin defects of immunoincompetent rats. Either immediately or after a waiting period of 1 (group 1) or 2 (group 2) weeks, the matrices were covered with neonatal epidermis (Fig. 1 and Table 1) and then assessed after 7, 14 and 21 days. After 21 days, the matrices were excised for histological analysis.

Matrices

The two matrices, Matriderm® (Dr. Suwelack Skin & Health Care AG, Billerbeck, Germany) and Integra® (Integra Life Science, Plainsboro, NJ, USA), both 2 mm thick, were cut into pieces 2.5 cm in diameter to fit into the transplantation devices. After removal of the silicone foil of Integra®, both matrices were soaked for 30 min in Dulbecco modified Eagle medium (Invitrogen, Carlsbad, CA, USA) containing 10% fetal calf serum (Invitrogen). Immuno-incompetent female nu/nu rats, 6–8 weeks old (Elevage Janvier, Le Genet St Isle, France) were anaesthetised by inhalation of 5% Isofluran® (Baxter, Volketswil, Switzerland), and maintained by inhalation of 2.5% Isofluran® via mask. Before the operation, 0.5 mg/kg buprenorphine (Temgesic®, Essex, Luzern, Switzerland) for analgesia and retinol cream (Vitamin A ‘‘Blache’’®, Bausch & Lomb, Steinhausen, Switzerland) for eye protection were applied. To prevent wound closure from the surrounding skin, polypropylene rings, 2.6 cm in diameter, were designed in our laboratory. The rings were sutured into full-thickness skin defects created on the backs of the rats, using non-absorbable polyester sutures (Ethibond®, Ethicon, Piscataway, NJ, USA). The matrices were implanted within the polypropylene rings and covered with silicone foil (Silon-SES, BMS, Allentown, PA, USA). Finally, the rings were covered with 5 cm x 5 cm polyurethane sponges (Ligasano®, Ligamed, Innsbruck, Austria). No dressing changes were performed until epidermal grafting.

Neonatal rat epidermis

To obtain an epidermis free of dermal components, fullthickness skin samples of appropriate size from newborn Wistar rats (University of Zurich animal breeding

programme) were treated overnight in dispase (BD Biosciences, San Jose, CA, USA) diluted 1:1 in Hank's balanced salt solution (Invitrogen) containing 5 mg/ml gentamicin (Invitrogen) at 4 8C. The epidermis was peeled off the dermis immediately before transplantation. Neonatal epidermal grafts were transplanted onto both matrices either immediately or after a waiting period of 1 or 2 weeks. As a control, neonatal epidermis was also transplanted directly onto subcutaneous tissue. Subsequently, silicone foils and dressings were applied as described above. All experiments were repeated three to four times for both matrices and each waiting-period group (Table 1).

Photographic monitoring of take rates

Transplantation sites were photographically documented in a standardised way at 7, 14, and 21 days after transplantation of the neonatal epidermis. Images were exported to a graphic program (ImageJ1, http://rsb.info.nih.gov). Three independent observers delineated the area with intact epidermis (take). The enclosed area was calculated from the number of pixels in the delineated area compared with the number of pixels corresponding to the known diameter of the polypropylene ring [11]. The maximal area was 4 cm^2, which was therefore defined as 100% take rate.

Histology and measurement of neodermis

Wound sites were excised 21 days after epidermal grafting, embedded in O.C.T. compound (Tissue-Tek®, Sakura Finetek, Japan), and stored at -80 ℃. Cry osections (12 mm thick) from the central portion of the excised area were prepared and stained with haematoxylin and eosin (Sigma, St. Louis, MO, USA) and thereafter mounted within Eukitt® (Fluka, Buchs, Switzerland). The thickness of the neodermis (defined as the vertical distance between the basal layer of the epidermis and the subcutaneous adipose tissue) was measured four times in each of five representative sections cut from each sample.

Immunohistochemistry

For immunostaining vascular endothelial cells, frozen sections were fixed and permeabilised for 5 min in acetone/ methanol (1:1) at -20 ℃. After blocking with 3%

bovine serum albumin (Sigma) in phosphate-buffered saline (Invitrogen), sections were incubated with an anti-rat CD31 antibody (BD Pharmingen, San Jose, CA, USA) conjugated previously with the Zenon Alexa Fluor 555 labelling kit (Molecular Probes,
Eugene, OR, USA). Hoechst 33342 (Sigma) was used for nuclear counterstaining. Sections were mounted with fluorescent mounting medium (DakoCytomation, Glostrup, Denmark).

Statistical analysis

The results were analysed using VassarStats® online software (http://faculty.vassar.edu/lowry/VassarStats.html) performing unpaired Student's t-test or one-way analysis of variance where appropriate; $p < 0.05$ was considered significant.

Results

Take rates of epidermal grafts

The mean take rate of neonatal rat epidermis 7 days after transplantation on a group 1 wound bed was 20% (±13.2%) for Matriderm® using 3 rats and 13% (±3.8%) for Integra® using 3 rats (Figs. 2 and 3). The mean take rate of neonatal rat epidermis 7 days after transplantation on a group 2 wound bed was 49% (±8.5%) for Matriderm® using 4 rats and 52% (±16.0%) for Integra® using 4 rats (Figs. 2 and 3). There was no statistically significant difference between the take rates of Matriderm® and Integra® within the same waiting period after matrix implantation (group 1: $p = 0.45$; group 2: $p = 0.66$). There was, however, a clear difference in take rates, which depended on how long the matrices were implanted before epidermal transplantation (Fig. 3) ($p < 0.05$). A second series of macroscopic inspections was undertaken 14 days after epidermal transplantation onto group 1 and group 2 wound beds. Again, the take rates on group 1 matrices were significantly lower, namely 20% (±10.5%) for Matriderm® and 14% (±1.4%) for Integra® (Figs. 4 and 5; $p = 0.82$), compared with group 2 rates which were 33% (±7.6%) for Matriderm® and 48% (±19.0%) for Integra® (Figs. 4 and 5; $p = 0.23$). The situation in terms of take rates remained basically unchanged 21 days after transplantation of the epidermis. Therefore, it can be assumed that a stable incorporation was achieved. At any observed time point after transplantation of the epidermis, the take rates of the matrices with a waiting period of 2 weeks were significantly superior to the take rates of the matrices with a waiting period of 1 week ($p < 0.05$).

Neodermis measurement

According to the experimental scheme shown in Fig. 1, the dermo-epidermal grafts were excised 21 days after epidermal transplantation. The mean thickness of the neodermis was 1.7mmfor both Integra® (±0.2 mm) and Matriderm® (±0.1 mm) (Figs. 6 and 7); after transplantation without any matrix was it significantly lower at 0.7 mm (±0.3 mm), using 4 rats in each case (Fig. 7).

Vascularisation

The qualitative immunohistochemical analysis of vascularisation 3 weeks after epidermal grafting revealed no obvious difference between epidermis derived from Matriderm® or from Integra® (Fig. 8).

Discussion

To our knowledge, this is the first study comparing Matriderm® and the dermal part of Integra® (i.e. Integra® without silicone foil) in an animal model. We did not find significant differences between the two matrices regarding graft take, neodermis formation or vascularisation. Importantly, there was also no difference in the take rates when the epidermis of neonatal rats was transplanted onto the neodermal tissue derived from the tested matrices. The following aspects deserve a more detailed comment. For proper comparison, we used a 2-mm thick Matriderm® that equalled the thickness of the dermal part of Integra®. With respect to physiological skin dimensions and eventual scarring, it appeared reasonable to use dermal templates approximating to the physiological thickness of human dermis. In all previous reports, Matriderm® was used in a thickness of 1 mm [12,14,15]; the thinner product allows simultaneous matrix implantation and split-thickness skin grafting. However, this one-step approach is debatable since it does not appear to yield better long-term results than split thickness skin grafting alone [13]. Another methodological novelty was the application of pure neonatal epidermis for grafting. This approach enabled us to monitor the quality and function of the developing neodermis without the influence of variable amounts of vascularised native dermis present in split-thickness skin grafts.

The core finding of our study is that in this animal model Matriderm® and Integra® demonstrated similar performance with regard to incorporation into the wound, neodermal regeneration, vascularisation and, finally, acceptance of epidermal grafts. Matriderm® is composed of bovine dermal collagen I, III, V and elastin with no cross-linking [12,14,15], whereas Integra® consists of bovine tendon collagen type I and shark glycosaminoglycan, cross-linked with glutaraldehyde [1,3,16]. However, these structural and biochemical differences probably do not play an instrumental role regarding biological behaviour in a rat model. Alternatively, the higher resistance to degradation of Integra® due to cross-linking of collagen might be counterbalanced by other mechanisms, such as foreign body reaction [17,18]. Importantly, both matrices produced significantly more and better structured neodermis than occurred after epidermal grafting onto subcutaneous tissue. This is consistent with the hypothesis and some preliminary data in the literature, namely that using dermal templates results in better functional and cosmetic long-term outcomes than grafting only of split skin or cultured epidermis [3,4].

Furthermore, we generated compelling evidence that the time interval between matrix implantation and epidermal transplantation was important, in that take rates were significantly better ($p < 0.05$) when grafting was performed 2 weeks rather than 1 week after matrix implantation. This finding is no surprise, since we know from published clinical data [18], as well as from our own observations not yet published (of >50 paediatric cases), that a minimum of 3 weeks is required for Integra® to produce a neodermis suitable for epidermal coverage. As mentioned above, clinical or other experimental data using 2-mm thick Matriderm® are not available for comparison. Matrix vascularisation was addressed immunohistochemically by selectively staining vascular endothelial cells. Although analyses were done qualitatively and not quantitatively, histological comparison demonstrated approximately the same vascularisation pattern in both matrices. The fact that matrix and graft takes were identical for both templates strongly corroborates the thesis that a very similar, if not identical, blood supply is present.

A point deliberately not addressed was the problem of scarring and contraction. Notoriously, all dermal replacement materials lead to some scar formation and thus they contract, usually more than full-thickness skin contracts [6]. Although we did observe such shrinking in this study, we opted against studying the issue. Rat models in general and, in particular, the use of polypropylene rings create a non-natural situation and thus a hardly controllable bias. On the other hand, the great advantage of the ring was to prevent wound closure from the surrounding tissue, i.e. host cell migration from the wound margins.

Conclusion

Standardised comparison in a rat model of Matriderm® and the dermal part of Integra® revealed no statistically significant differences in their biological properties or behaviour. In contrast, the time span between matrix implantation and final coverage was crucial with respect to matrix incorporation and subsequent epidermal grafting. Randomised controlled human studies are needed to ultimately define the roles of these matrices in skin replacement strategies.

Conflict of interest

All authors disclose any financial and personal relationships with other people or organisations that could inappropriately influence (bias) their work. Particularly, no employment, consultancies, stock ownership, honoraria, paid expert testimony, patent applications/registrations and grants or other funding were received in the context of this work.

Acknowledgements

The authors thank Lina M. Nilsson, ETHZ, for her critical comments and help with editing, and Stefan Oberhaensli, ETHZ, for his suggestions regarding statistics. Samples of Matriderm® and Integra® dermal regeneration templates were kindly donated. Role of the funding source: No external funding existed for this study. All co-workers were employed by the University of Zurich or University Children Hospital of Zurich.

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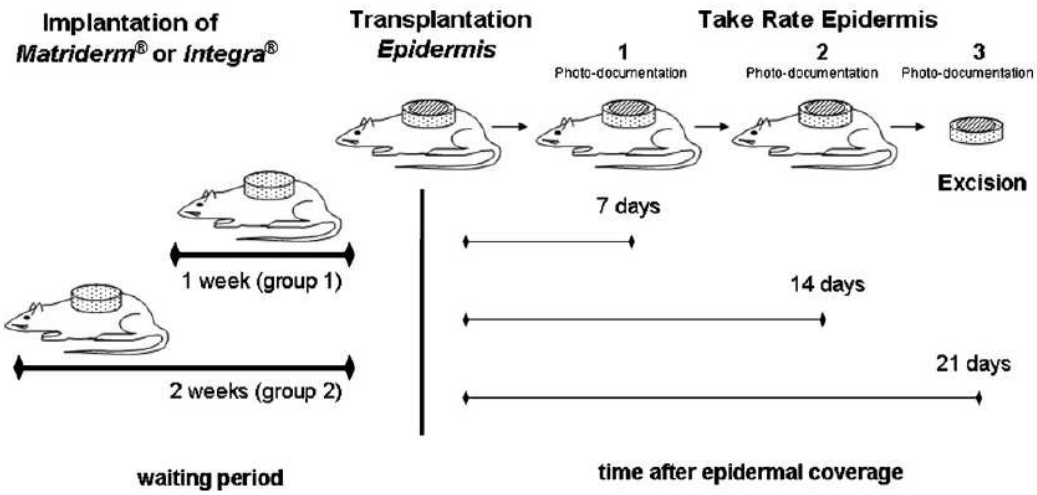

Fig. 1 – Schematic overview of experiments.

Table 1.

Table 1 – Matrix used, waiting period before graft, number of animals per group.

Matrix	Waiting period (weeks)	Number of animals
Matriderm®	1	3
Integra®	1	3
Matriderm®	2	4
Integra®	2	4
None (control)	0	4

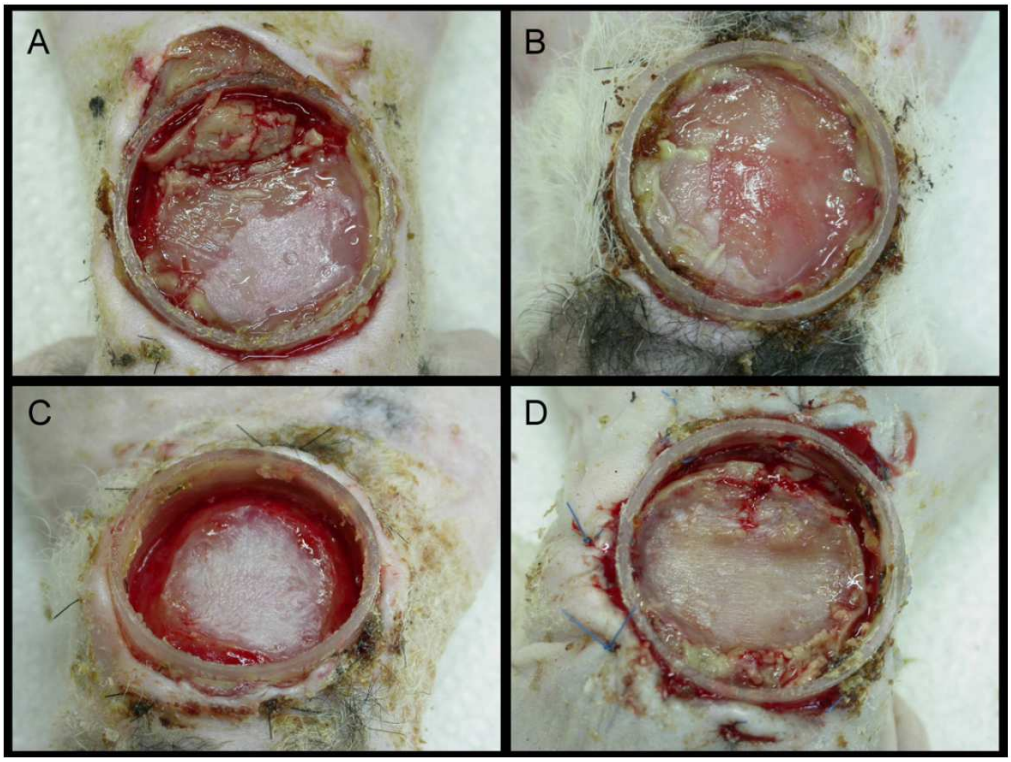

Fig. 2 – Photodocumentation 7 days after transplantation of neonatal epidermis: (A) Matriderm® implanted for 1 week (group 1), (B) Integra® implanted for 1 week (group 1), (C) Matriderm® implanted for 2 weeks (group 2) and (D) Integra® implanted for 2 weeks (group 2).

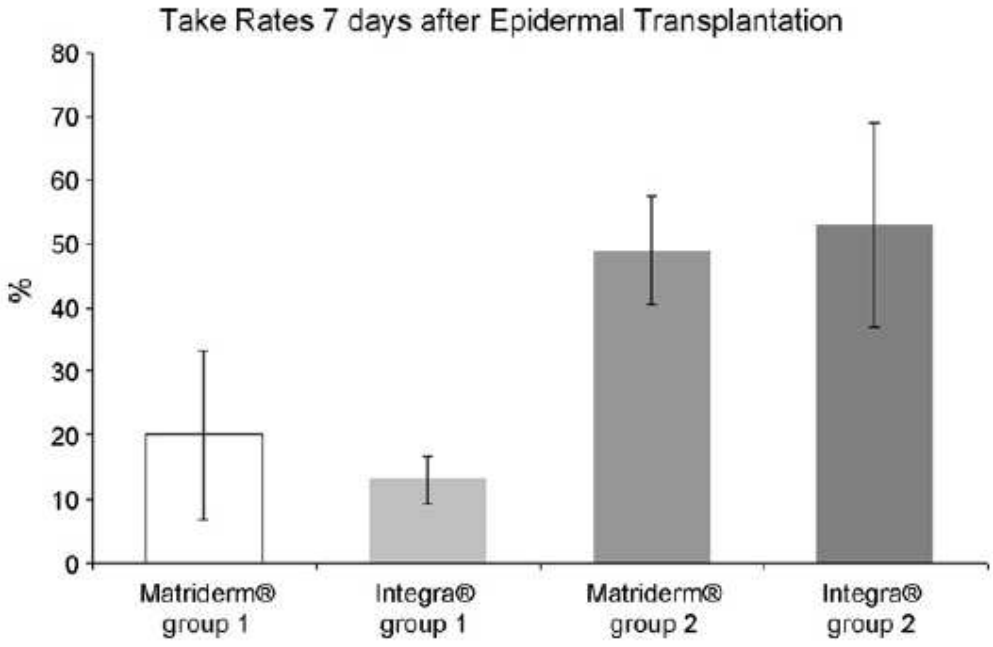

Fig. 3 – Mean take rates of epidermis 7 days after transplantation onto matrices.

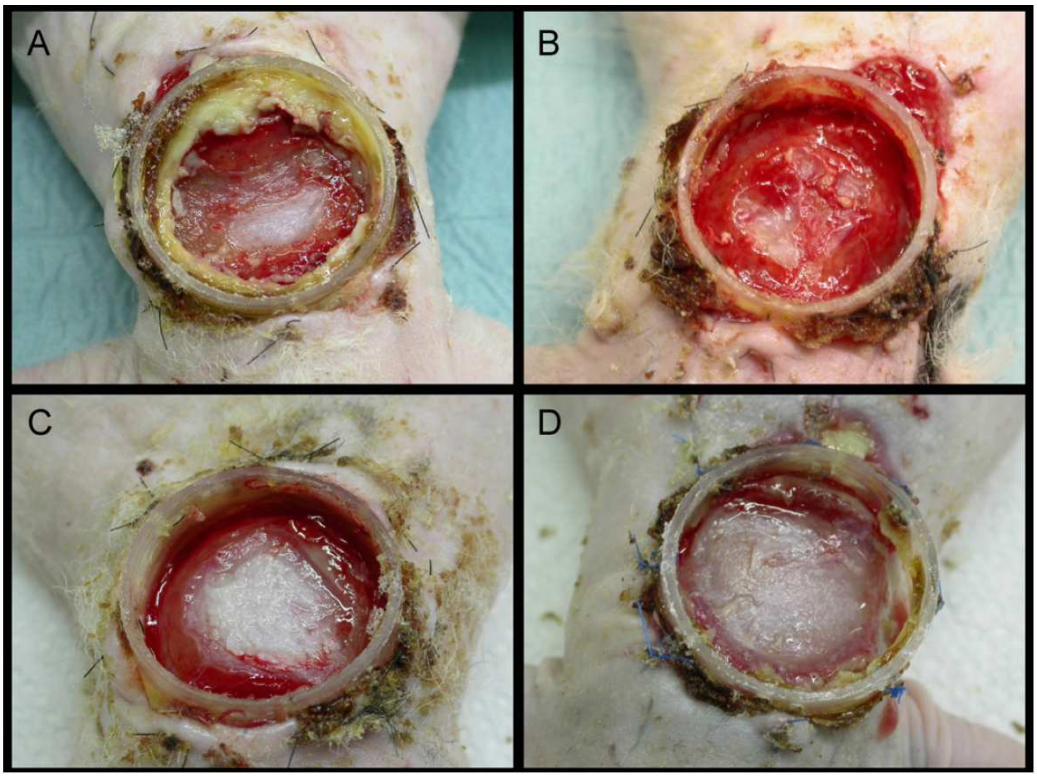

Fig. 4 – Photodocumentation 14 days after transplantation of neonatal epidermis: (A) Matriderm® implanted for 1 week (group 1), (B) Integra® implanted for 1 week (group 1), (C) Matriderm® implanted for 2 weeks (group 2) and (D) Integra® implanted for 2 weeks (group 2).

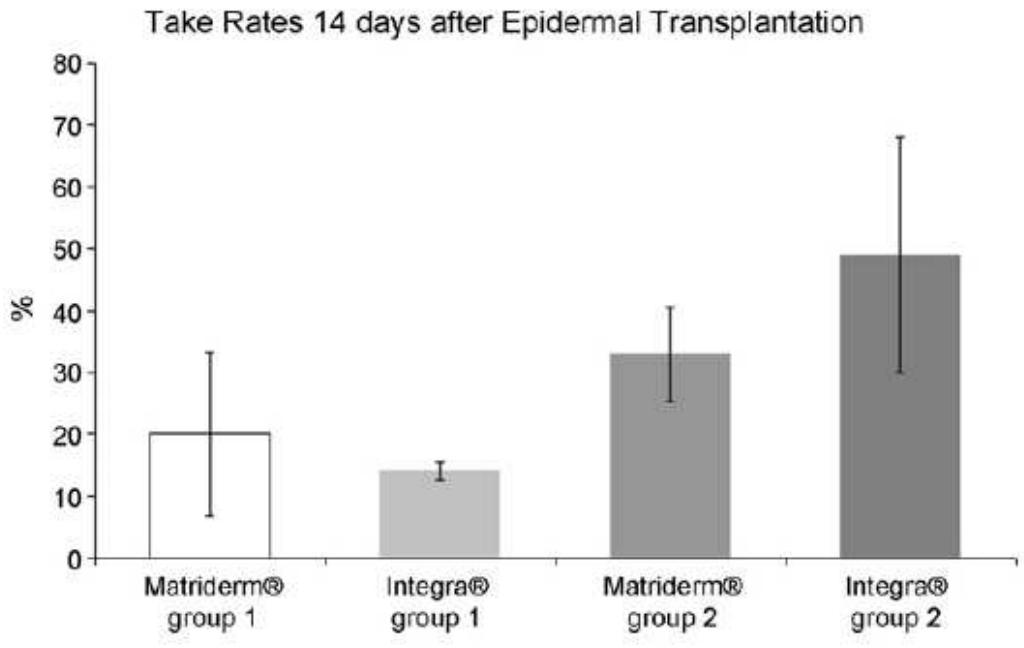

Fig. 5 – Mean take rates of epidermis 14 days after transplantation onto matrices.

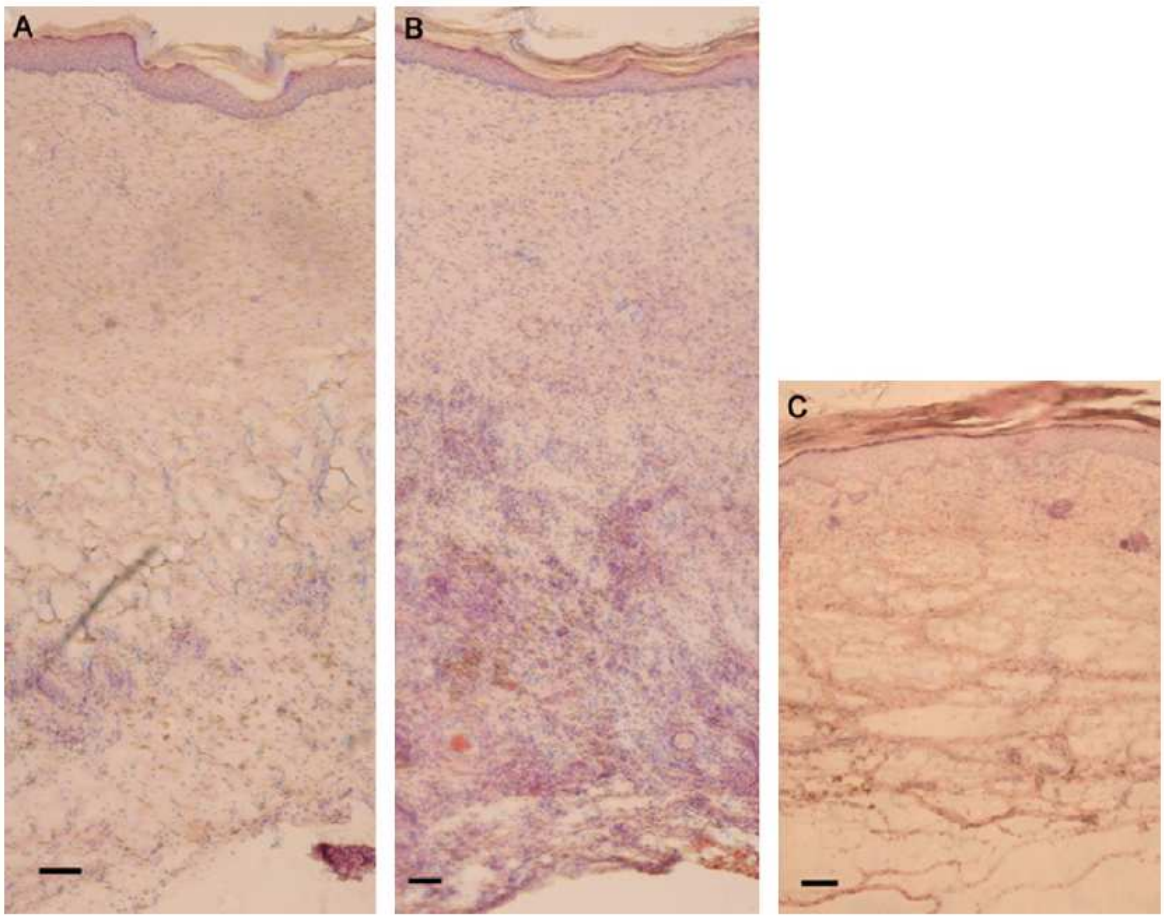

Fig. 6 – Haematoxylin and eosin staining of neodermis 21 days after transplantation of epidermis, group 2: (A) Integra®, (B) Matriderm® and (C) control, direct transplantation of the epidermis onto subcutaneous tissue (scale bar: 100 mm).

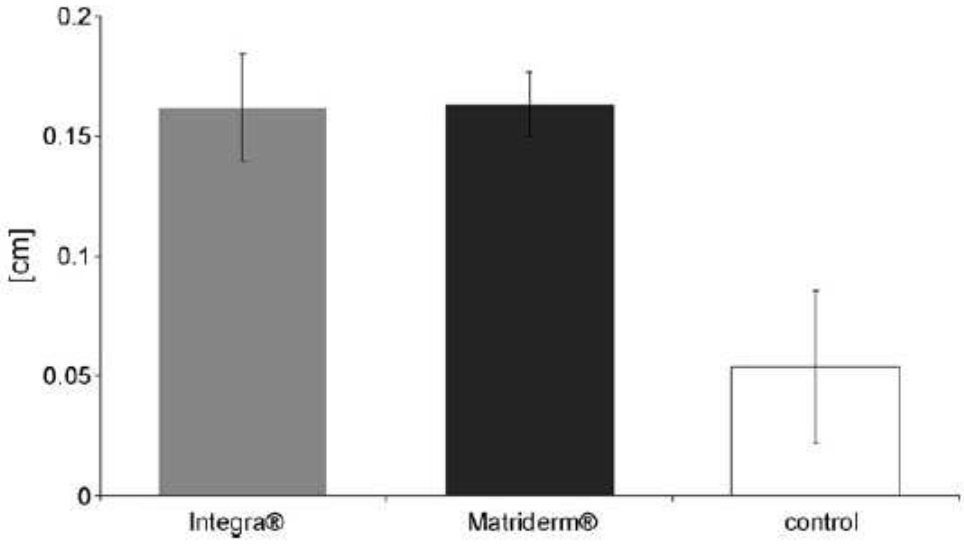

Fig. 7 – Mean thickness of the neodermis 21 days after transplantation of the epidermis onto Integra®, Matriderm® and directly onto the subcutaneous tissue (control).

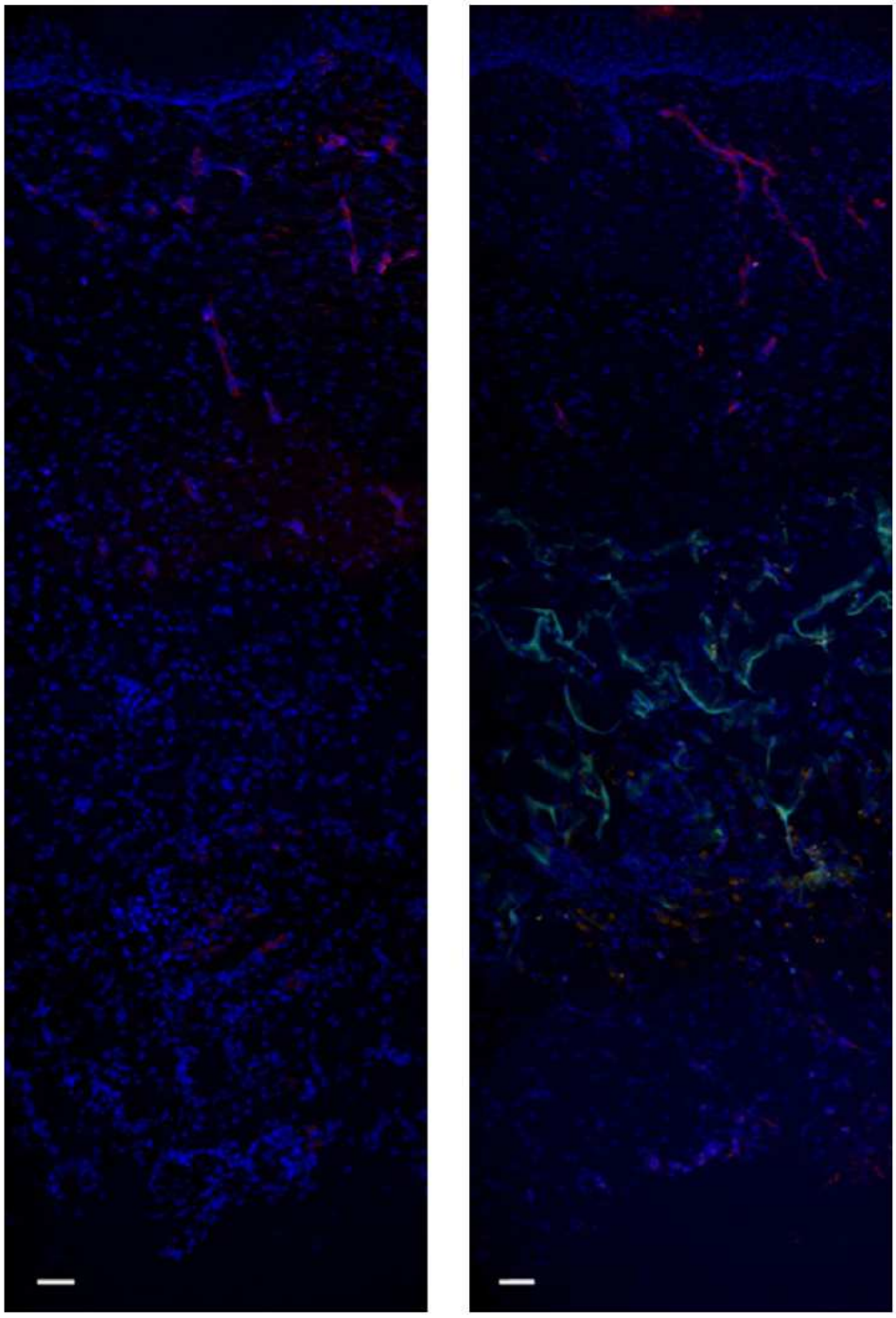

Fig. 8 – Histology of neovascularisation: Left, Matriderm®. Right, Integra®. Red, CD 31 antibody staining. Blue, Hoechst staining. Green, autofluorescence of collagen in Integra®. No autofluorescence is observed with Matriderm® (scale bar: 50 mm).

2.4 Tissue engineering of skin

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Abstract

The engineering of skin substitutes and their application on human patients has become a reality. However, cell biologists, biochemists, technical engineers, and surgeons are still struggling with the generation of complex skin substitutes that can readily be transplanted in large quantities, possibly in only one surgical intervention and without significant scarring. Constructing a dermo-epidermal substitute that rapidly vascularizes, optimally supports a stratifying epidermal graft on a biodegradable matrix, and that can be conveniently handled by the surgeon, is now the ambitious goal. After all, this goal has to be reached coping with strict safety requirements and the harsh rules of the economic market.

Introduction

Large full-thickness skin defects resulting from burns, soft tissue trauma, congenital giant nevi, and disease leading to skin necrosis [1], [2], [3], [4], [5], [6], [7] and [8], represent a significant clinical problem that is far from being solved. The main challenges encountered are the following two.

First, there is donor site shortage for autologous skin transplantation when the defect exceeds 50–60% of the total body surface area (TBSA) [9]. The typical clinical example is a massive deep burn.

Second, most conventional skin grafting techniques to provide autologous defect coverage are based on transplanting split-thickness skin (the today's gold standard). Split-thickness skin contains all of the epidermis but only part of the dermis, and that frequently leads to scarring. Rarely, scarring is mild and irrelevant. Often, particularly in children, there is hypertrophic scarring or keloid formation that is frequently disabling and disfiguring [10] and [11]. Interestingly, full-thickness skin transplantation is not usually associated with scarring, however autologous full-thickness skin transfer can only be performed for injured areas <2% TBSA.

Theoretically, both main problems, donor site shortage and scarring, could be reduced if not eliminated if it were possible to grow an autologous full-thickness skin analogue with near normal anatomical and functional properties: a cultured dermo-epidermal skin substitute.

There are still some major challenges concerning the development of such a skin substitute:

1) A dermo-epidermal substitute should exhibit a barrier function immediately or rapidly after transplantation. A central question that remains is what minimum level of differentiation (and barrier function) has to be achieved in a skin substitute during its growth in vitro to result in an optimal structure and function after its transplantation onto the patient.

2) On its way to an optimized and long lasting structure and function, a dermo-epidermal substitute should be efficiently and appropriately vascularized. Attempts to

reach this goal have entered a period of significant progress; however, a final breakthrough is still missing.

3) Much is still unknown about the mechanisms by which tissues form and heal, yet insights from developmental biology and other biological disciplines are already guiding the development of “intelligent materials” that work with nature's own mechanisms of organogenesis and repair. Biologically active and appropriate matrices and factors in combination with automated (tissue printing) techniques [12], designed to produce a new generation of complex skin substitutes both, in a desired number and with a constant quality, are now the guidelines of modern “skingineering”.

This article summarizes the progress in the field, reviews some critical aspects of the underlying cell and skin biology, and points out some remaining challenges, key clinical landmarks, commercial considerations and future directions that may foster the progress in the engineering of skin.

Principles of skin reconstitution and scarring

Skin is an efficient barrier against external influences such as mechanical disturbances, UV radiation, pathogenic microbial agents; it prevents the substantial loss of body fluid and plays a significant role in thermoregulation and immune defence. Different cell types, such as keratinocytes, melanocytes, fibroblasts and endothelial cells, constituting the epidermis, the dermis and the subcutis, are necessary to guarantee its full function (Fig. 1). If this barrier is destroyed, different measures need to be taken, depending on the depth of the skin defect. Injuries involving the epidermis alone, and injuries extending into the superficial layer of the dermis, will re-develop an epidermis without surgical intervention, provided there is a sufficient number of keratinocyte stem cells present in the remaining epidermis or in the residual dermis. If epidermal keratinocytes are missing, regeneration may be achieved by epithelial stem cells derived from hair follicles and/or sweat glands.

Patients with defects extending into the deeper dermis or even the hypodermis need a more complex treatment, as the injured surface is depleted of its keratinocyte stem cells. In this case the “gold standard” approach is to apply split-thickness skin grafts that contain all the epidermis and marginal parts of the dermis, thereby transferring

self-renewing keratinocyte stem cells to the affected area. With full-thickness skin lesions, involvement of surgical excision with closure of the wound is almost always mandatory and scarring a fact [13]. Hence, next to the availability of stem cells, scarring is another crucial hallmark of skin reconstitution. Because scar formation is unique to humans in most aspects, animal model research is only of limited value and has not contributed much to our understanding and the treatment of scars. To mend the damage, the body has to produce new collagen fibers. However, because the body cannot re-build the tissue exactly as it was, the new (scar) tissue will have a distinct collagen pattern and a different skin texture (and quality) than the surrounding normal skin.

The main feature of the phenomenon “scarring” is called “wound contraction”. Wound contraction is caused, at least in part, by the presence of myofibroblasts which develop characteristics of smooth muscle cells under the influence of TGFβs [14]. In contrast to embryonic wound healing [15] and [16], which still occurs without scarring, wound contraction is the general mode of wound healing in humans after birth [17] and [16]. Taken together it can be said that successful reconstitution of skin with skin substitutes depends crucially on two factors: the presence of self-renewing keratinocyte stem cells for re-epithelialization, and a functional dermal substitute consisting of the appropriate cellular and acellular components, that allow no or only limited scarring of the developing skin.

Key events in the development of skin substitutes

The first milestones in skin research and skin tissue engineering were the enzymatic separation of the epidermis and dermis [18] and the in vitro culture of keratinocytes [19]. A major breakthrough was made in 1975, when Rheinwald and Green managed to grow human primary epidermal cells in serial culture on a layer of lethally irradiated 3T3 murine fibroblasts (Fig. 2). These researchers showed that limitations observed previously in the cultivation of epidermal cells in surface cultures were not intrinsic, but due to the complex relationship between keratinocytes and fibroblasts [20]. Taking advantage of the 3T3 cell-feeder layer technique, an epidermal graft could be expanded to more than 500 times its size within 3–4 weeks [21]. After the first clinical application [22], cultured epidermal autografts (CEAs) were tested in almost all leading burn centers world wide [23], [24], [25], [26], [27], [28], [29] and

[30]. However, there were disadvantages that included a widely variable CEA take, depending on the wound site and status, age of the patient, and knowledge and experience of the operator [26] and [31]. In 1981 Bell et al. [32] generated a dermo-epidermal substitute which was tested in an animal model. Subsequently this technique was transformed into the product Apligraf®, which was prepared using human allogeneic fibroblasts and keratinocytes (see also Table 1). On the basis of this development also dermo-epidermal skin substitutes consisting of human autologous keratinocytes and fibroblasts in bovine collagen were applied in severe burn patients [33]. Interestingly, because of the effort to establish dermo-epidermal skin grafts, researchers revealed the importance of epithelial–mesenchymal interactions. They observed that the cross-talk between fibroblasts and keratinocytes was essential for the establishment of a functional basement membrane [34] and [35]. A further significant progress was the development of a bilayered “artificial skin” [36]. This acellular collagen-glycosaminoglycan-based skin substitute is now commercially available as Integra Artificial Skin® (commonly referred to as Integra). Integra® was developed in the 1980s and commercially launched in the United States in 1996. The appealing idea of combining cultured keratinocytes with Integra® has generated a fascinating new field of research and much optimism to finally provide patients with a dermis off the shelf and a laboratory grown epidermis, thus eliminating the need for donor sites. However, reality has shown that simple cultured epidermal autografts do not take well on the neodermis produced by Integra® [37] and [38].

The invention of Integra Artificial Skin was certainly a major step in tissue engineering of skin. However, the time has come to explore new avenues for the development of a single step clinical application of novel dermo-epidermal skin substitutes. This not only because of increasing hospitalization costs (increasing health insurance costs, budget restrictions) but also, and predominantly, to optimize clinical skin substitution with superior functional and cosmetic results.

Improved engineered skin substitutes for clinical applications

Two main groups of patients would profit most from a tissue-engineered skin substitute. The first group includes burn patients, suffering from an acute life-threatening situation (Fig. 3A). Large and deep burn wounds leave little remaining healthy skin to be used for split-thickness skin grafts. The challenge here is to rapidly produce large quantities of autologous, dermo-epidermal substitutes. The second

group denotes the elective or chronic situation. Disabling scars, giant nevi (Fig. 3B) or chronic ulcers are ideally replaced by a skin graft of matching size, texture, and colour. Full- and split-thickness skin grafts may not always be available in a sufficient quantity. The challenge in this respect is the engineering of functionally and cosmetically adjusted skin substitutes, ready for transplantation at a previously scheduled point in time.

There exist several “commercial” treatment modalities next to skin grafting. These may or may not help to improve the structure and function of the grafted skin, such as some dermal substitutes or keratinocyte sprays. In any case, various basic problems are still encountered.

For a given skin substitute to attach promptly after transplantation, a well prepared and vascularized wound bed is required. This is not always easy to achieve in deep burn wounds or with chronic wounds. If a dermal substitute reaches a threshold thickness [39], vascularization is too slow to assure nutrition of the overlying epidermis resulting in epidermal necrosis or graft loss. Therefore, most dermal substitutes thicker than 1 mm (Integra®, Matriderm®) are applied using a two-step approach. This avoids epidermal necrosis, as the dermal substitute is given sufficient time to vascularize. However, an additional operation is needed for transplantation of an epidermal component. This procedure is lengthy, gives no guarantee of success and is an additional stress factor for the patient. The transplanted epidermal component produces skin of varying quality and exhibits properties that are distinct from the original. Features of the transplant may be missing elasticity, contraction of the graft, lack of pigmentation, and thereby lack of protection against UV radiation. All these factors let us conclude that there is still a high potential for the development of novel, significantly improved skin substitutes.

Tissue homeostasis, keratinocyte stem cells, and rapid vascularization as indicators of skin quality

To reconstruct skin in the laboratory, a skin biopsy from the patient is required to isolate the different cell types (Fig. 4). Mainly, keratinocytes, melanocytes, dermal fibroblasts and vascular endothelial cells can be obtained. Each cell type is expanded in its appropriate culture medium, in close contact with appropriate extracellular matrix components, and if required in the presence of supportive (mesenchymal)

cells. As a result a complex skin substitute may develop. However, the quality of such a skin graft is frequently not determined prior to transplantation. How can the quality of a given engineered skin substitute be monitored? To date only a very limited number of indicators or markers has proven useful in this respect. Cytokeratin 19 (K19) expressed in basal keratinocytes (Fig. 5A) has recently been described as a marker for epidermal homeostasis, and as an indicator of young, possibly laterally expanding skin [40]. Furthermore, K19 is expressed in the stratum basale of engineered skin substitutes, indicating a potentially thriving and functional epidermis that is very likely to be taken after its transplantation. However, as K19-positive keratinocytes are frequently described as keratinocyte stem cells [41] and [40] and as they are no longer detectable in human individuals older than 2 years, it remains to be determined whether K19-positive basal keratinocytes are a subtype of keratinocyte stem cells, exclusively expressed in very young skin. Other keratinocyte stem cell markers may be the integrin α6 chain (highly expressed) in combination with the transferrin receptor, CD71 (low expression) [42] and [43]. The problem with any of these markers is that their usefulness has to be confirmed by a reliable bioassay. The only reliable bioassay, however, appears to us is the formation of a stratified epidermis that remains fully functional for at least 12 weeks after transplantation. An additional criterion significantly influencing the quality of a given skin substitute is its rapid and appropriate vascularization after transplantation. The thickness of a (non-pre-vascularized) skin graft that can easily become vascularized is about 0.7–1.0 mm. In grafts thicker than 1.0 mm, new blood vessels cannot grow quickly enough to nourish the overlaying epidermal layer [44]. Vascularization is particularly problematic if the respective skin substitute is based on a dense or highly cross-linked extracellular matrix, or if it is placed over fatty tissue or poorly vascularized wound beds. One promising line of research investigates the pre-vascularization of engineered tissues in vitro [44], [45], [46], [47], [48] and [49].

In this context adult human dermal microvascular endothelial cells (HuMECs) are submerged within fibrin or collagen hydrogels in which they develop into true 3D capillaries (Fig. 5B) displaying a real lumen (Fig. 5C) [50]. Subsequently these pre-vascularized substitutes are transplanted to have the engineered capillaries stabilized by mural cells (pericytes, smooth muscle cells) derived from the wound bed. Finally the engineered capillaries are thought to connect to the microvessels of

the wound ground (inosculation). So far only very few laboratories managed to convincingly show the proof of this principle.

What has been achieved so far: currently available skin replacements

Several commercial products were developed during the last 30 years. Most of them were designed for permanent use, some of them as temporary substitutes. They contain cells of differing origin (autologous, allogenic or xenogeneic) and biodegradable materials (naturally occurring or synthetic polymers) as scaffolds for cell attachment and facilitated handling. To give an overview, we have classified them into epidermal, dermal and dermo-epidermal substitutes (Table 1).

Epidermal substitutes contain autologous keratinocytes, often grown in the presence of murine fibroblasts. Most products belong to the category of “cultured epidermal autografts”, also called CEAs (Epicel®, Epidex™, Myskin™), for which keratinocytes are grown to stratified cell sheets. Starting off from a skin biopsy, the production of the final substitute takes about 3 weeks [51] and [52]. This is why burn wounds initially need to be treated with temporary wound dressings. Once applied to the pre-treated dermal wound bed, CEAs can be applied directly or in combination with other methods (e.g. the sandwich technique). Several studies and multi-centre trials over the past years [52] and [53] show a wide range of take rates with an average value of 50% or less [54] and statements about qualitative outcomes are inconclusive due to the diversity of application methods. Disadvantages are mainly their slow preparation time, variable engraftment rates, difficult handling due to the thin, fragile cellular layers and their high production costs [30].

Another approach for epidermal cellular replacement is the use of cultured autologous keratinocytes in suspension (ReCell®) [55], [56] and [57]. With this method, keratinocytes can be sprayed onto the wound bed directly after having been prepared from a biopsy in the operation room. Although this method has shown a somewhat faster epithelialization and epidermal maturation in wound models [58], this method is not suited to treat 3rd degree burn wounds. The quality and patient's benefit of this method in clinical settings is still debated.

As with these methods a dermal component is missing, the degree of epidermal attachment and scarring is highly dependant on the quality and condition of the underlying dermal wound bed [39]. The additional unsatisfying results in regard to mechanical stability and scarring led to another approach in skin substitute development.

Engineered *dermal* substitutes restore dermal tissue by promoting new tissue growth and optimising healing conditions [59]. They need to be covered by a permanent epidermal surface or substitute. Some of them consist of acellular matrices and are permanently incorporated into the patient's wound bed (AlloDerm®, Integra®, Matriderm®) [60], [61], [62], [63], [64] and [65]. After application to a prepared dermis, these substitutes are colonized and vascularized by the underlying cells [66]. Finally an autologous neodermis is formed. As soon as vascularization has advanced sufficiently (usually 3–4 weeks after application), a split-thickness skin graft can be placed on the neodermis [67]. Others include human allogeneic cells and are applied as transient wound dressings that stimulate wound healing (Dermagraft®). Dermagraft® consists of allogeneic fibroblasts seeded in a polyglactin mesh [68]. They secrete growth factors and deposit dermal matrix proteins and are thought to facilitate the healing process [59], [67] and [69]. Histologic evaluation of biopsies did not show any evidence of immunologic response [70], however, the product is mostly used for chronic venous or diabetic foot ulcers [69].

The application of dermal substitutes using the two-step surgical procedure has shown improved scarring [62], but more recent approaches are utilizing thinner dermal layers, with the aim of transplanting the dermal substitute together with the epidermal graft in a single step [56], [63], [64], [71] and [72]. Clinical studies have so far shown promising results with a thin (1 mm) dermal matrix (Matriderm®) limited to the hand and wrist region. Further clinical applications and studies will show if this method can be applied more broadly.

Few engineered, “off-the-shelf” *dermo-epidermal* substitutes have been produced. Human allogeneic neonatal keratinocytes and fibroblasts are combined with a scaffold to form a temporary covering (Apligraf®, Orcel®) and are used mostly for chronic wounds [39], [59], [66] and [69]. Studies have mostly reported about its use for chronic wounds and ulcers and have shown a higher incidence of wound closure

[74] and [75]. One study reports improved clinical outcome [76] for burn patients. For autologous cultured dermo-epidermal substitutes [59], keratinocytes and fibroblasts are collected from a burned patient's biopsy and added to a collagen-glycosaminoglycan substrate [77]. Cultivation time after biopsy needs about 4 weeks before the substitute can be transplanted. Few clinical trials have been conducted so far. In terms of graft take and scar appearance, results appear superior to conventional techniques [59], but further clinical studies need to confirm these results.

Commercial considerations

Tissue engineering of skin was thought to have such obvious clinical benefits that it led to unrealistic clinical and commercial expectations. The initial objective with the aim to rapidly come up with perfect skin grafts for patient treatment soon turned out to be unrealistic. Delivering a customized product shows a high complexity from its fabrication with autologous cells until delivery at its destination. In addition to slow progress for graft development, even slower progress for administrative demands like quality and safety controls and regulations (GMP production, FDA approval, microbiologic and immunologic safety required) might lead to a delayed market launch. Needless to say that a relatively small number of customized products, with already high production costs, raises costs even more and reduces the chance for health care institutions to approve of upcoming costs. All in all, development and production of tissue-engineered skin substitutes seems unattractive so that companies deviate towards more broadly applicable products, such as acellular materials or temporary dressings, which can be sold as “off-the-shelf” products [78] and [79].

It should, however, be taken into consideration that the application of an engineered substitute made of autologous cells in a fitting size and of matching colour and texture, would also simplify medical treatment. Multiple operations could be avoided by applying the substitute in a one-step procedure. Some corrective operations would become unnecessary, and therapy for chronic diseases could find an easier solution. With fewer operations and interventions, medical risks for patients could be reduced, recovery time decreased and costs thereby be diminished. In addition to making treatment methods easier and optimizing the biology and function of skin substitutes, hopes for a satisfactory cosmetic outcome are high.

Concluding remarks and perspectives

Tissue engineering of skin is based on 25 years of research and rests on a strong background of material technologies and cell and molecular biology. The challenge that still remains is the generation of a complex dermo-epidermal substitute that can be securely and conveniently transplanted with minimal scarring in one single surgical intervention. Means to significantly speed up vascularization in these complex skin grafts, such as controlled release of angiogenic and/or vasculogenic factors from matrices [80], [81], [82], [83], [84] and [85], seeding endothelial cells directly into the matrix, and engineering the vasculature directly into the tissue [86], [87] and [88] will largely contribute to reach this goal.

Furthermore the development of novel ECM matrices and scaffolds will still be central in engineering optimized skin grafts. Numerous attempts to develop novel skin substitutes are still (and will be in the future) based on purified ECM components such as collagens, fibrin and hyaluronic acid (see also Table 1). These represent a relative basic ECM environment, which is conductive to the activities of determined, non-stem keratinocytes. Imposing a tissue-specific identity on the epidermal keratinocyte stem cell fraction is likely to require more specific influences during their 3D organotypic culture and after transplantation on the organism, if not already during their expansion in 2D cultures. It is promising that the outcome of growth factor administration can be improved enormously with the employment of technically relatively simple slow release schemes [89]. However, it also needs to be taken into account that an epithelium and its mesenchyme support each other in an equilibrated and complete manner. Thus, the growth factors and matrix components released by different, but interacting cell types may be even more “instructive” than slowly released “instructive” factors and matrices. It remains to be investigated whether purified factors alone or distinct cell types in concert, or combinations thereof, will provide the “skingineering” concept of the future [39], [89] and [90].

The field of stem cell biology also has to be integrated in this future concept. There is a huge potential for using human originated adult stem cells as a source of in vitro generation of skin [91], [92] and [93]. Human keratinocytes derived from the epidermal stratum basale [20], [21], [22], [42] and [43], from hair follicles [94], [95], [96] and [97] and as recently suggested, also from eccrine sweat glands [98] and

[99], are sources that allow scaling up the self-renewing keratinocyte fraction in engineered human skin grafts.

The experience of the past 25 years has identified that in the production of tissue-engineered materials the focus must move more quickly from laboratory to clinical use. Yet safety precautions have to be met. Production costs must be carefully considered, so that reimbursement gives a sufficient income. New products have to be more rapidly adapted to a rapidly changing regulatory environment. Despite initial unrealistic commercial and clinical expectations, tissue-engineered skin has already now delivered considerable benefits to patients with burns, accidents, infections and chronic wounds. “Skingineering” has enormous potential that has just begun to be realized.

Conflict of interest

The authors have declared that no conflict of interest exists.

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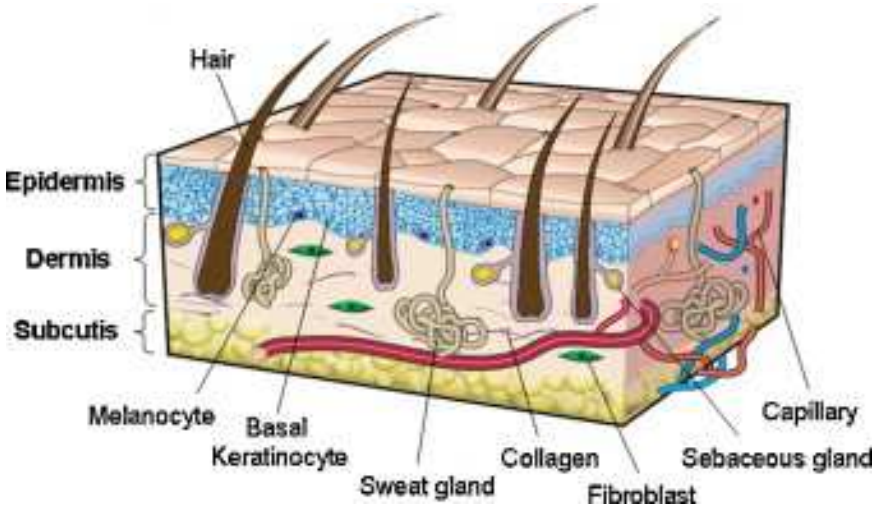

Fig. 1. The structure of human skin. Human skin consists of three layers. The outermost layer, the *epidermis*, measures about 0.1–0.2 mm. Keratinocytes and melanocytes are prominent cell types of the epidermis. The basement membrane physically separates the epidermis from the underlying *dermis*, however, functionally it connects both tissues. The major cellular components of the dermis are fibroblasts producing extracellular matrix. The rich dermal vascular system provides all cell types with nutrients. Skin appendages like hair follicles, sebaceous and sweat glands are situated throughout the dermis and epidermis. The *subcutis* (*hypodermis*) consists mainly of fat tissue, which functions as insulation and as an energy source [16].

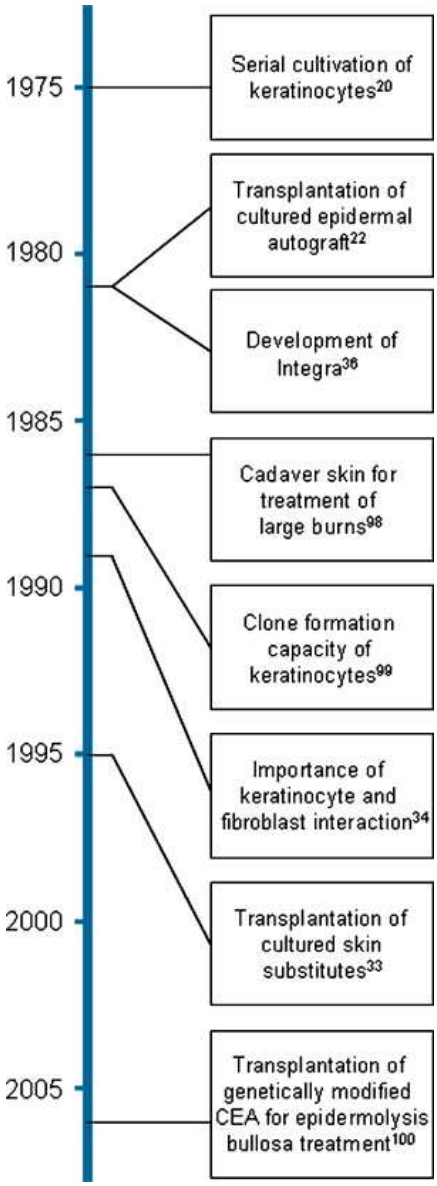

Fig. 2. Timeline of key developments [100], [101] and [102].

Table 1. Examples of commercially available skin substitutes.

Table 1 – Examples of commercially available skin substitutes.

	Commercial product	Company	Layers	References
Cellular epidermal replacement	Epicel®	Genzyme Corp.	Cultured epidermal autograft (autologous keratinocytes grown in the presence of murine fibroblasts)	[26,103]
	Epidex™	Euroderm GmbH	Cultured epidermal autograft (autologous outer root sheet hair follicle cells)	[104–106]
	Myskin™	Celltran Ltd.	Cultured epidermal autograft (autologous keratinocytes grown in the presence of irradiated murine fibroblasts)	[107–109]
	ReCell®	Clinical Cell Culture (C3), Ltd.	Autologous epidermal cell suspension	[55,110,111]
Engineered dermal substitute	AlloDerm®	LifeCell Corp.	Acellular donated allograft human dermis	[112–115]
	Dermagraft®	Advanced BioHealing Inc.	Bioabsorbable polyglactin mesh scaffold seeded with human allogeneic neonatal fibroblasts	[67,68,116]
	Integra®	Integra LifeSciences Corp.	Thin polysiloxane (silicone) layer; cross-linked bovine tendon collagen type I and shark glycosaminoglycan (chondroitin-6-sulfate)	[8,62,65]
	Matriderm®	Dr. Suwelack Skin & Health Care AG	Bovine dermal collagen type I, III, V and elastin	[63,73,117]
Engineered dermo-epidermal substitutes	Apligraf®	Organogenesis Inc.	Human allogeneic neonatal keratinocytes; bovine collagen type I containing human allogeneic neonatal fibroblasts	[74–76,118]
	OrCel®	Forticell Bioscience, Inc.	Human allogeneic neonatal keratinocytes on gel-coated non-porous side of sponge; bovine collagen sponge containing human allogeneic neonatal fibroblasts	[119–122]

Fig. 3. The need of tissue-engineered skin. (A) The acute situation. Patient, suffering from large and deep burn injury after escharatomy, necessitating necrectomy and skin coverage. Due to the large total body surface area affected, little healthy skin remains as donor site for a split-thickness skin graft. (B) The elective situation. Patient with a giant congenital melanocytic nevus, expanding over the elbow. After resection, the patient would benefit of the application of a tissue-engineered skin substitute of matching colour and texture to the adjacent skin.

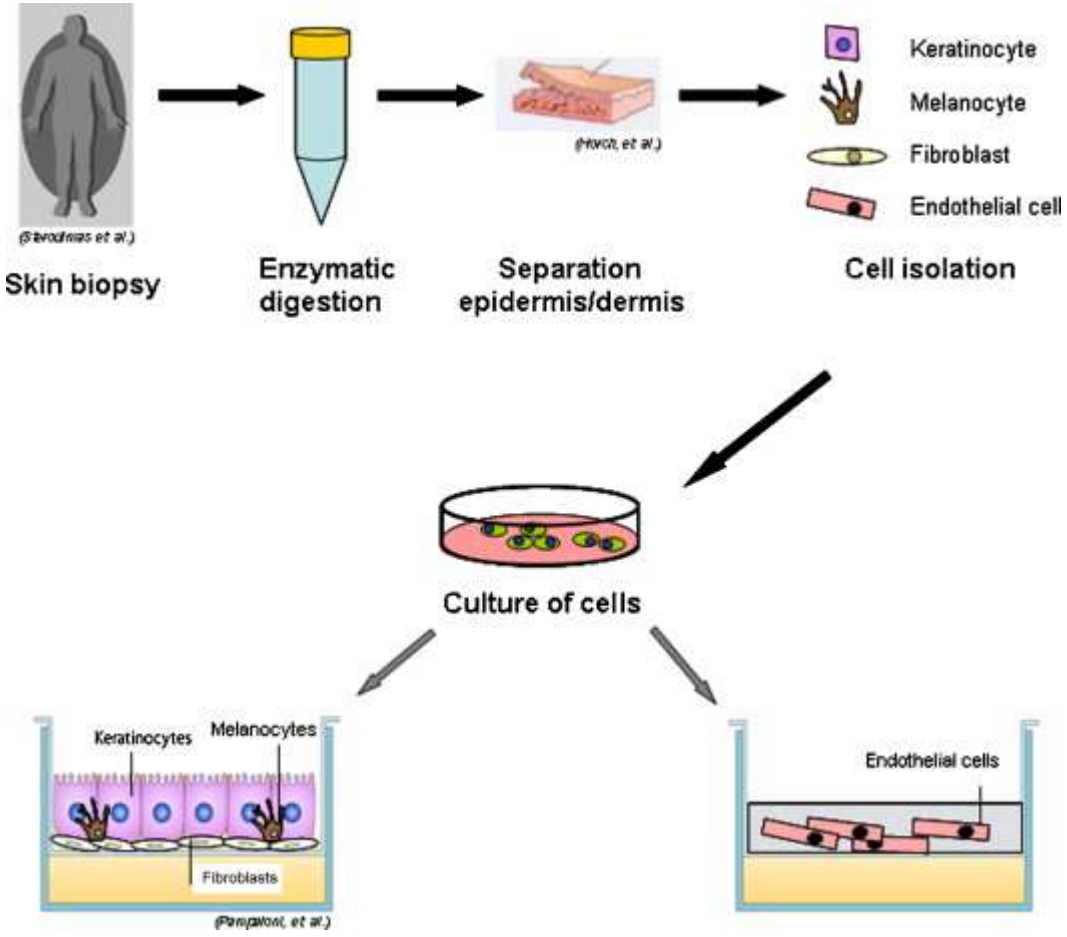

Fig. 4. Tissue engineering of the skin. Reconstruction of tissue-engineered skin. A patient's skin biopsy is treated enzymatically to digest the basement membrane. Epidermis and dermis are separated, followed by isolation of epidermal keratinocytes and melanocytes, dermal fibroblasts and vascular endothelial cells. The cells are cultured in culture dishes in the appropriate medium for each cell type. Dermo-epidermal substitutes, consisting of fibroblasts in a collagen hydrogel and keratinocytes/melanocytes seeded on top of the hydrogel. Cultured vascular endothelial cells are seeded in hydrogels to form a network of lumenized capillaries [50], [54], [123] and [124].

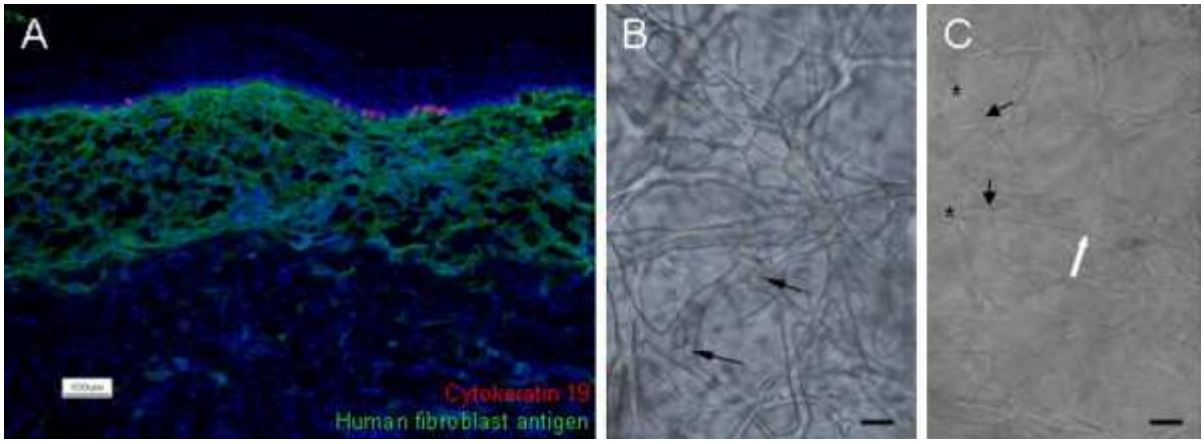

Fig. 5. Epidermal homeostasis in transplanted dermo-epidermal substitutes and pre-vascularized hydrogels. (A) Cytokeratin 19-positive (K-19) human keratinocytes are indicative of an intact epidermal homeostasis in a grafted dermo-epidermal skin substitute. The dermo-epidermal skin substitute consisting of human keratinocytes as epidermal equivalent and a collagen type-I hydrogel containing human dermal fibroblasts was transplanted onto the back of immuno-incompetent rats. Cytokeratin 19-positive cells (red) are organized in clusters. Note that Cytokeratin 19-positive cells are restricted to the well-defined stratum basale. Human dermal fibroblasts (green) are recognized by the human CD90 (Thy-1)-specific antibody, whereas the rat tissue underneath stains negatively (cell nuclei: blue) [40]. (B and C) Phase contrast micrograph of capillary formation *in vitro*. Human microvascular endothelial cells (HuMECs) were isolated from a skin biopsy, cultured and seeded in a hydrogel. (B) HuMECs develop into dense networks of branching solid cords after 1 week. Anastomoses are indicated by black arrows. (C) Branching, completely lumenized capillaries developing in hydrogels (white arrow). Regularly alternating nuclei (black arrows) and the cytoplasm are retracted towards the plasma membrane. The two branches of a capillary are indicated by asterisks [50]. Scale bars: (A) 100 μm; (B and C) 50 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

3 Conclusion

The idea that human eccrine sweat glands contain epidermal keratinocyte stem cells was discussed in several previous reports, however, data and a final proof were not reported (Brouard, 2003, Barrandon, 2007, Nakamura, 2009).

In this work I could show, to my knowledge for the first time, that isolated primary human eccrine sweat gland cells are capable to develop into a stratified epidermis both, in vitro and in vivo. Notably, there was no major difference between sweat gland cells and epidermal keratinocytes regarding the formation of an epidermis.

The following aspects deserve a detailed comment. In all our analyses, sweat gland cells forming the epidermis, expressed well-known interfollicular epidermal markers such as integrin α6β4, involucrin, loricrin, envoplakin and transglutaminases (Biedermann, 2010, Pontiggia, 2009, Tharakan, 2010). Although we observed a somewhat delayed expression of several of these markers in sweat gland cell derived epidermises (such as K2e expression), these finally developed into a complete and functional epidermis. The delay mentioned above is most likely due to sweat gland cells undergoing a phenotypic transition from ductal or secretory glandular cells into interfollicular epidermal keratinocytes.

The sweat gland cell transition may represent the reversion of the ontogenetic process in embryogenesis, when ectodermal cells differentiate into ductal and secretory cells (Fu, 2005). Two conceivable mechanisms for this cell transition may exist:

1. Sweat gland cells might de-differentiate (or retrodifferentiate) into a more naive state, after which they differentiate into a new phenotype, i.e., epidermal keratinocytes.
2. A multipotent, sweat gland-derived stem cell population may differentiate into epidermal keratinocytes in response to their new epidermal environment.

Based on the fact that the new sweat gland derived epidermis was permanently maintained in vivo, it has to be postulated that self-renewing keratinocytes assured the integrity of the new epidermis. It may well be possible that even both, sweat gland derived epithelial stem cells and re-programmed sweat gland derived cells in concert formed the new epidermis.

Published data show that epithelial stem cells, located in hair follicles can re-establish the epidermis in a wound situation (Ito, 2005, Levy, 2005). However, there is also glabrous skin (palms and soles) of humans in which not a single hair follicle can be found, but a relatively great number of sweat glands is present (Frinkel, 2001). Therefore, our findings support the idea that sweat glands, in analogy to the hair follicle bulge, contain potential keratinocyte stem cells (Brouard, 2003, Barrandon, 2007, Nakamura, 2009), which have the potential to reconstitute human epidermis upon wounding or even to permanently participate in regenerating human interfollicular epidermis.

This concept is supported by our observation that the epidermis formed by sweat gland cells had no morphological and structural features of palmo-plantar or buccal epithelia, but features of normal interfollicular epidermis. This is in contrast to a report by Miller et al., 1998, who showed that porcine sweat gland cells developed an epidermis displaying palmo-plantar features. This different finding is most likely due to the fact that porcine skin harbors apocrine glands (which most likely do not even function as sweat glands), whereas human skin harbors functionally different eccrine sweat glands (Montagna, 1964, Ferry, 1995).

A point which should be addressed in subsequent studies is the question where the epithelial stem cell niche is located in the sweat gland. The knowledge in this respect is very limited (Barrandon, 2007, Nakamura, 2009). One report suggests that keratinocyte stem cells are located in the proximal intraglandular duct immediately adjacent to the secretory domain or in the upper ductal portion (Langbein, 2005). Our data suggest that stem cells are situated in the secretory portion (Pontiggia, manuscript in preparation).

The finding that sweat gland-derived epithelial cells obviously represent an additional source of keratinocytes which can grow to a near-normal autologous epidermis is important from a tissue engineering point of view. It is particularly relevant for patients requiring large and urgent covering of skin defects, such as severe burn injuries (third degree; >50% body surface area). In these cases, self-renewing keratinocytes are urgently required for the in vitro production of as many life-saving skin grafts as possible.

I was also involved in a second work in which we tested for potential keratinocyte stem cell markers and for markers of epidermal homeostasis as signs for the quality of produced human dermo-epidermal skin substitutes (Pontiggia, 2009).
The novelty we observed in these experiments is the expression pattern of the cyto-keratins K15/K19. Interfollicular keratinocytes neither express K15 after isolation from skin samples and growth on cell culture plastic, nor is K15 detectable in dermo-epidermal substitutes in vitro. Obviously, keratinocytes on cell culture plastic and within engineered skin substitutes display a wound situation in which tissue homeostasis is not at all, or only imperfectly established. In contrast, after transplantation of engineered skin constructs onto immunoincompetent rats, we were able to identify K15-positive keratinocytes in the basal layer of the epidermis. Furthermore, we observed that K19 is expressed in single cells or in clusters of cells in the basal layer of the epidermis after transplantation of skin substitutes. We suggest therefore that K19-positive cells represent a population of keratinocytes that is adapted to lateral expansion of the epidermis.
Taken together, we propose that basal K15/19-positive keratinocytes in the basal layer are indicators of epidermal homeostasis, thus a marker for the quality of (grafted) engineered dermo-epidermal substitutes.

Dermo-epidermal skin substitutes can also be used to study certain skin diseases. Although several methods to study melanoma biology are known, a reliable model to study the genesis of melanoma in human skin in vivo is missing (Satyamoorthy, 1999, Bissell, 2010, Verbridge, 2010).
With our dermo-epidermal skin substitutes we can also employ human melanoma cells in the epidermal portion. The engineered skin constructs are then transplanted onto athymic rats. Using this in vivo assay we can show the process of basement membrane penetration, migration of melanoma cells into the dermis and formation of metastasis. As a methodological novelty our humanized 3-dimensional in vivo model can open new prospects in basic melanoma research (Biedermann, manuscript in preparation).

An issue often underestimated is the generation of a proper dermal substitute. In the 1970’s and 1980’s the focus in skin tissue engineering was the epidermis. It was a

challenging task to isolate and culture human epidermal keratinocytes (Rheinwald, 1975), even more to transplant the first keratinocyte sheets to patients (O'Connor, 1981, see also for review Böttcher, 2010). But soon it was recognized that not only the epidermis is important. Wound contraction and scarring are dependent on the dermis (Grinnell, 1994, Gurtner, 2008, for review Böttcher, 2010). This is supported by the observation that functional dermal substitutes, used in combination with epidermal tissue in surgical skin reconstruction, resulted in a significantly better functional and cosmetical outcome, as compared to grafting of only cultured epidermal sheets or split-thickness skin (Moiemen, 2001, Moiemen, 2006).

This prompted us to employ commercially available matrices to engineer dermo-epidermal skin grafts (Schneider, 2009). We found that dermal templates based on lyophilized collagen "sponges" are not suitable to produce an autologous skin substitute. Of course, these matrices may serve as a basis for the development of a vascularized neodermis in clinical use. In a second surgical intervention then this neodermis may be covered by split-thickness skin grafts (for review, van der Veen, 2010, Böttcher, 2010). We also realized this quality in our studies, showing that the formation of an epidermis is possible after vascularization of collageneous templates, such as Integra® or Matriderm® (Schneider, 2009).

However, we showed that our dermo-epidermal skin grafts, based on collagen hydrogels, are perfectly suited to bring about a high quality, novel skin in a one step operation. The biological properties of these skin grafts do greatly support the formation of an epidermis and a well-vascularized dermis after transplantation (Biedermann, 2010, Pontiggia, 2009). Importantly, the biological advantages of these hydrogels can now be combined with plastic compression of hydrogels (Braziulis, manuscript in preparation) and the use of a biodegradable supporting mesh (Hartmann, manuscript in preparation) to yield a more stable construct. This prevents the skin substitutes from contraction and leads to a significant improvement in handling properties, especially concerning a future surgical application. Thus, our challenging aim to produce a large-scale autologous dermo-epidermal skin substitute for clinical application is in reach.

Of course, skin constructs have to be tested in preclinical studies before there is an implementation for humans.

The typical in vivo model for preclinical studies for new skin products is to apply them onto pigs (Sullivan, 2001, Middelkoop, 2004). At the moment we conduct such a preclinical study. We have already established a pig model for our large (7 x 7cm) skin grafts, which has not been described before (Schiestl, manuscript in preparation).

Taken together it can be said that the tissue engineering of skin is the point of intersection of applied and basic research. This is also underscored in my work. On the one hand, I was able to show that eccrine sweat gland cells are capable to form a stratified, functional epidermis. On the other hand I was involved in improving our dermo-epidermal skin substitutes and in conducting a preclinical study. All this is done to apply autologous skin equivalents onto patients in the near future.

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5 Curriculum Vitae

Full name	Thomas Biedermann
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Education

Since 2004	**PhD thesis**, Group of PD Dr. Ernst Reichmann, University Children's Hospital Zurich, Switzerland
1999 - 2004	**Study of Biochemistry, University Leipzig, Germany** (diploma thesis: „Die Rolle von GDNF und GFRαs während der Neurogenese: Entwicklung neuer neuronaler Screeningsysteme", supervision Prof. Dr. Andrea A. Robitzki)
1992 - 1998	Johann Mathesius Gymnasium Rochlitz, Germany (Degree: Abitur)

Presentations and Posters

December 2005	7th International EMBL PhD Student Symposium, Heidelberg, Germany Poster: Genetically engineered dermo-epidermal skin substitutes
September 2007	12th Congress of the European Burns Association (EBA), Budapest, Hungary Presentation: Identifying Self Renewing Keratinocytes in Human Skin and Engineered Skin Substitutes
June 2008	Annual Tissue-Engineering-and-Regenerative-Medicine-International-Society-(TERMIS) European-Chapter Meeting, Porto, Portugal Poster: Keratinocytes derived from human eccrine sweat glands
October 2008	5th World Congress of European Club for Pediatric Burns, Gdansk, Poland Presentation: Matriderm versus Integra: an Experimental Comparative Study Workshop organization and supervison: Ratskingineering – Rat Model in Tissue Research

September 2009 13th Congress of the European Burns Association (EBA), Lausanne, Switzerland
Poster: Matriderm versus Integra: an Experimental Comparative Study

Others

November 2005 participant in patenting course “Embracing life – challenges and questions in patenting for life sciences”, Forum for Genetic Research of the Swiss Academy of Sciences, Gwatt, Switzerland

May 2006 Local Organizing Committee Member, “Denkmal Muttermal Kongress”, University Childrens Hospital Zurich

Further education

July 2005 LTK1 - Introductory Course in Laboratory Animal Science

November 2009 LTK2 - Education for persons responsible for directing animal experiments

6 Publications

Biedermann T, Pontiggia L, Böttcher-Haberzeth S, Tharakan S, Braziulis E, Schiestl C, Meuli M, Reichmann E, Human eccrine sweat gland cells can reconstitute a stratified epidermis, *J Invest Dermatol,* in press, doi:10.1038/jid.2010.83

Böttcher-Haberzeth S, **Biedermann T**, Reichmann E, Tissue engineering of skin, *Burns*, Review, 2010, 36(4):450-460

Tharakan S, Pontiggia L, **Biedermann T**, Böttcher-Haberzeth S, Schiestl C, Reichmann E, Meuli M, Transglutaminases, involucrin, and loricrin as markers of epidermal differentiation in skin substitutes derived from human sweat gland cells, *Pediatr Surg Int,* 2010, 26(1):71-7

Montaño I, Schiestl C, Schneider J, Pontiggia L, Luginbühl JF, Böttcher S, **Biedermann T**, Braziulis E, Meuli M, Reichmann E, Formation of human capillaries in vitro: The engineering of pre-vascularized matrices, *Tissue Eng Part A*, 2010, 16(1):269-82

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Böttcher-Haberzeth S, **Biedermann T**, Reichmann E, Wege zu einer neuen Haut: Von den zellbiologischen Grundlagen über Tissue Engineering zu einem neuen Hautsubstitut, Paediatrica, 2009, 20(4):57-9

Rothermel A, **Biedermann T**, Weigel W, Kurz R, Rüffer M, Layer PG, Robitzki AA Artificial design of 3D retina-like tissue from dissociated cells of the mammalian retina by rotation-mediated cell aggregation, *Tissue Eng,* 2005, 11(11-12):1749-1756

In preparation

Kiowski G, **Biedermann T,** Civenni G, Dummer R, Sommer L, Reichmann E, In vivo reconstitution of human melanoma in dermo-epidermal skin grafts, (in preparation)

Braziulis E, Diezi M, **Biedermann T**, Pontiggia L, Schmucki M, Hartmann F, Luginbühl J, Meuli M, Reichmann E, Compressed collagen gels for large scale engineered skin transplants, (in preparation)

Biedermann T, Böttcher-Haberzeth S, Pontiggia L, Braziulis E, Schiestl C, Meuli M, Reichmann E, Human sweat gland derived cells and melanocytes interact in dermo-epidermal skin substitutes, (in preparation)

Böttcher-Haberzeth S, **Biedermann T,** Schiestl C, Hartmann F, Schneider J, Meuli M, Reichmann E, Artifical dermal skin substitutes compared in a single step transplantation assay, (in preparation)

7 Contributions

I contributed to the following publications as indicated:

Human Eccrine Sweat Gland Cells Can Reconstitute a Stratified Epidermis

Macroscopic and histologic appearance of SdES and KdES *in vitro* and after transplantation onto immuno-incompetent rats (**Figure 1 e-h**)

Tissue specific markers in KdES and SdES (**Figure 2 e-h**)

Preparations of human eccrine sweat gland epithelial cells are not contaminated by basal epidermal cells (**Figure 3 a-f**)

Epidermal stratification and homeostasis in KdES and SdES after transplantation onto immuno-incompetent rats (**Figure 4 a-m**)

Epidermal stratification and homeostasis in KdES and SdES after transplantation onto immuno-incompetent rats (**Figure 5 a-m**)

Markers to Evaluate the Quality and Self-Renewing Potential of Engineered Human Skin Substitutes In Vitro and after Transplantation

Quantification of K19-expressing cells (**Figure 4 d**)

Transplantation of dermo-epidermal substitute (**Figure 6 a**)

Processing and staining of transplanted graft (**Figure 6 b – g**)

Quantification of Ki67/K19 positive cells (**Figure 6 h**)

Matriderm versus Integra: a Comparative Experimental Study

Schematic overview of experiments (**Figure 1, Table 1**)

Transplantation of grafts (**Figure 2**)

Take rate measurement (**Figure 3**)

Transplantation of grafts (**Figure 4**)

Take rate measurement (**Figure 5**)

H/E staining of grafts (**Figure 6**)

Neodermis measurement (**Figure 7**)

Histology of neovascularization (**Figure 8**)

Tissue Engineering of Skin (review)

Text parts:

Key events in the development of skin substitutes

Tissue homeostasis, keratinocyte stem cells, and rapid vascularization as indicators of skin quality

Figures and Tables

The structure of human skin (**Figure 1**)

Timeline of key developments (**Figure 2**)

Examples of commercially available skin substitutes (**Table 1**)

Tissue engineering of the skin (**Figure 4**)

Epidermal homeostasis in transplanted dermo-epidermal substitutes and pre-vascularized hydrogels (**Figure 5 A**)

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